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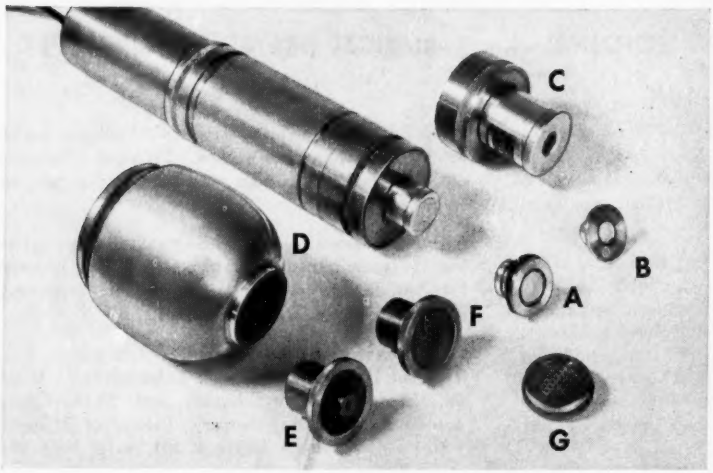

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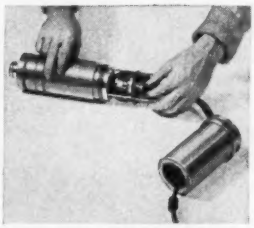

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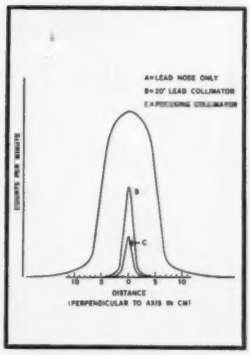

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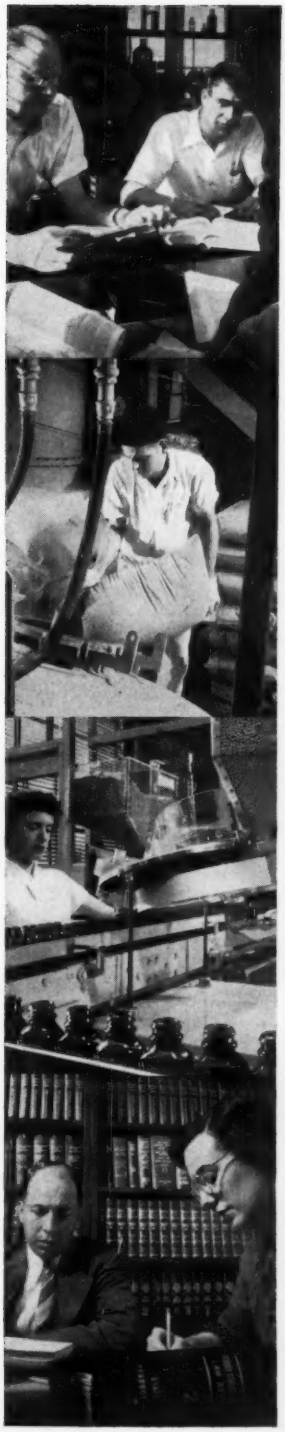

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BIOCOLLOIDS OF URINE IN HEALTH AND IN CALCULOUS DISEASE. III. THE MUCOPROTEIN MATRIX OF URINARY CALCULI [1]

By WILLIAM H. BOYCE and NORMAN M. SULKIN

(From the Departments of Urology and Anatomy, Bowman Gray School of Medicine of Wake Forest College, Winston-Salem, N. C.)

(Submitted for publication March 19, 1956; accepted May 24, 1956)

Over a period of three years 676 renal or ureteral calculi have been decalcified by chelating agents. The matrix has been recovered by ultrafiltration through collodion membranes and subjected to analysis by a variety of techniques. The present report is concerned with the microscopic structure and the histochemical properties of the matrix recovered from calcigerous stones.

MATERIAL AND METHODS

Preparation of matrix for histochemical studies. The decalcifying solution contained 5 per cent (wt/v) tetrasodium ethylenediaminetetraacetate and 10 per cent (v/v) formalin adjusted to pH 7.8 ± 0.1 with 12 N HCl.

Sixty-four calcigerous stones of wet weight 60 mg. to 28.35 gm., when surgically removed from the renal pelvis or ureter, were immediately rinsed in water and bisected with a hacksaw. The two halves were placed in a stoppered Erlenmeyer flask containing 500 ml. of decalcifying solution. The filings from the sawcut were subjected to chemical (1) and spectroscopic (2) analyses.

The flasks containing the halved calculi were carefully stored at 3°C. and every effort was made to prevent agitation of the solution. At 7 to 10-day intervals the decalcifying solution was removed by pipette and filtered through collodion membranes of 0.01 micron in pore size.[3] Refiltration through membranes of average pore diameter less than 0.005 micron did not result in recovery of additional material. The decalcifying solution was carefully replaced and the process continued for 3 to 27 weeks, depending upon the size of the stone and the rate of decalcification. The residual matrix invariably formed a complete cast of the original stone, and decalcification of a degree permitting sectioning with the microtome was evidenced by translucence when a light was held beneath the flask. In many instances no gross evidence of decalcification was observed until the entire "stone" was found floating on the surface of the solution.

Only a very minute amount of material was recovered from the ultrafiltration of the decalcifying solution through collodion membranes. This was recovered by ethanol-ether dissolution of the membranes at minus 5° C, and the mucoprotein precipitate was concentrated by centrifugation. After dehydration and embedding, this material was sectioned and treated in the same manner as the residual matrix.

When decalcification was complete the matrix was gently floated into smaller containers, dehydrated and embedded in the manner usually employed for microscopic study of tissues. Serial sections 8 to 10 microns in thickness were taken at intervals through the matrix of each calculus. Representative sections from each specimen were then treated according to the techniques described below.

Three calculi in this group of 64 formalin fixed stones had a centrally calcified core with an enveloping surface of laminated organic material 4 to 12 mm. in thickness (Figure 1). A portion of this surface material was cut away, fixed in formalin, sectioned with the microtome, and treated by the methods described below. These calculi, with most of the roentgenolucent cover intact, were then decalcified and subsequently studied in the same manner as all other calculi in the series. The 24-hour urinary calcium excretion in each of these patients was less than 36 mg. on three or more determinations. This surface material was therefore considered to represent matrix that had not been calcified, and is subsequently referred to as uncalcified matrix in contradistinction to *matrix* which refers to the organic residue recovered by decalcification.

Hematoxylin and eosin. The acid hematoxylin eosin technique was employed in the same manner as for tissue staining (3, p. 114).

Periodic acid Schiff reaction. The McManus-Hotchkiss technique as described by (Lillie 3, p. 120–128) was utilized. Glycogen was removed from control sections by incubation in a buffered solution of 1 per cent malt diastase for one hour at 37° C. The sections were mounted on slides and impregnated with collodion. They were then treated with 1 per cent periodic acid for 10 minutes, stained in the Schiff reagent for 15 minutes and rinsed in sulphurous acid. For purposes of orientation, other sec-

[1] This investigation was supported by research grants A-259 from The National Institute of Arthritis and Metabolic Diseases, and B-342 from The National Institute of Neurological Diseases and Blindness, National Institutes of Health, Department of Health, Education, and Welfare, and by a grant-in-aid from the Squibb Institute for Medical Research, New Brunswick, New Jersey.

[3] Schleicher & Schuell Co., Kenne, N. H., "S & S Ultrafine Filters" the porosity of which has been checked by electronmicroscopy.

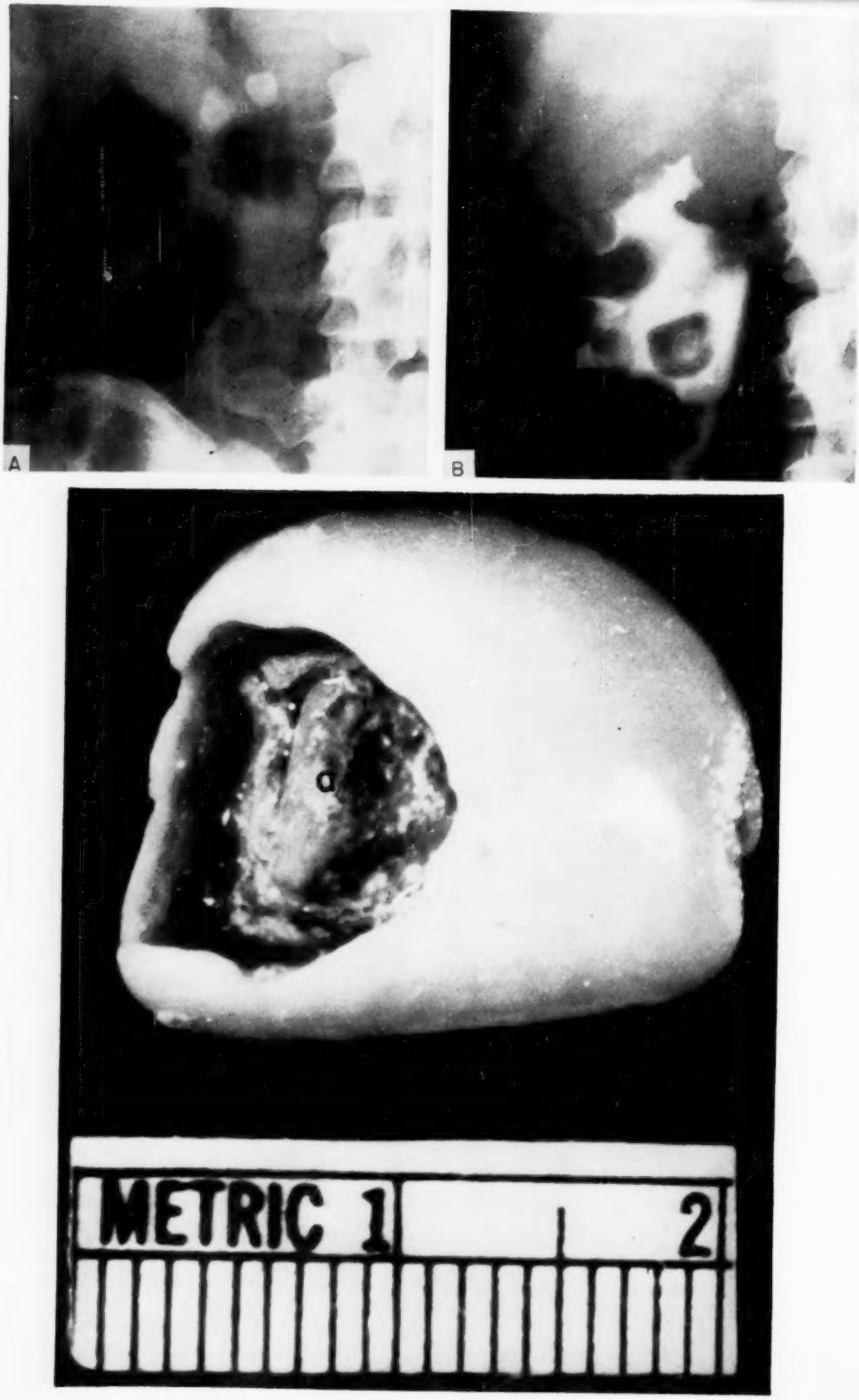

FIG. 1.

tions were studied by counterstaining with acid iron hematoxylin (Weigert's), and also without the preliminary incubation with diastase.

Aqueous toluidine blue. Holmgren's (4) technique for staining with 0.25 per cent aqueous solution of recrystallized toluidine blue was utilized. Comparable sections were also treated with concentrated sulphuric acid by previously described modifications of the Kramer-Windrum method (5). These sections were then stained with toluidine blue as above.

Sudan black. Paraffin sections were treated with sudan black and were mounted in glycerin jelly in order to determine whether the PAS positive reaction was due to the presence of partially oxidized lipids.

In addition to the above studies, sections from representative calcium oxalate, calcium phosphate, and mixed calcigerous stones, including those with magnesium ammonium phosphate, were stained with the von Kossa technique (3, p. 264), utilizing either safranin or PAS counterstain. Mayer's mucihematein and mucicarmine stains (3, p. 286) were also applied to sections from these calculi.

Matrix content of calculi. The halves of 264 freshly recovered calculi were grouped into four types of pools on the basis of chemical composition of the crystalline components. Pools of calcium phosphate, calcium oxalate, mixed calcium oxalate and phosphate, and mixed calculi containing magnesium ammonium phosphate were thus formed. The individual pools containing 25 to 100 calculi each were dried over "Drierite" in a vacuum desiccator at 20 C to constant weight. The final weight of the pools varied between 25.23 and 68.45 gm. The dried calculi were then decalcified by continuous agitation in 5 per cent ethylenediaminetetraacetate adjusted to pH 7.8, but without the addition of formalin. The solution was dialyzed against distilled water and ultrafiltered through collodion membranes. The matrix thus recovered was again dialyzed against distilled water and lyophilized. This matrix was dried *in vacuo* to constant weight and the percentage composition of the calculi determined.

Preparation of matrix for chemical analyses. Lyophilized matrix from the above pools was subjected to elemental analyses. Matrix from similar pools of 348 freshly recovered calculi which were not dehydrated were analyzed for protein and carbohydrate content. The Molisch and anthrone tests were performed as described by Dische (6). The orcinol (protein bound hexoses) and diphenylamine (sialic acid) tests were done as described by Winzler (7). The diphenylamine test was also applied by the method of Anderson and Maclagan (8) with control studies on urinary mucoprotein isolated by the techniques of these authors. The Hale test for acid mucopolysaccharides was performed by the method of Di Ferrante (9). The ninhydrin and biuret tests were made by standard techniques (10). Tests for hexuronic acids were made by the naphthoresorcinol (11) and carbazole (12) methods. Hexosamines were determined by both the Elson-Morgan (7) and indol-HCl (6) reactions. Control tests were made with standard solutions of human serum proteins, serum glycoproteins, hexoses, glucuronic, and galacturonic acid.

The elemental analyses of the lyophilized matrix were made by standard techniques (13). Analyses for carbon and hydrogen were by the universal tube filling method. Nitrogen determinations were by the Dumas procedure, and total phosphorus and sulphur determinations were by gravimetric analyses following a Carius wet digestion. The conjugated sulfates were determined by first precipitating the inorganic sulfates with barium chloride, followed by hydrolysis of the organic material in 2.4 N HCl for 3 hours and determination of the liberated sulfate by the method of Folin (14). The inorganic ash was determined by igniting the material in the presence of an excess of sulphuric acid.

RESULTS

Microscopic structure

The crystalline composition of the formalin fixed calculi was mixed calcium oxalate and calcium phosphate in 26, calcium oxalate in 18, calcium phosphate in 12, and calcium phosphate with magnesium ammonium phosphate in 8. It was not possible to detect any structural or histochemical characteristics of the matrix which could be correlated with the crystalline composition of any of these calculi, hence the following descriptions apply to any calculus in the series.

The organic matrix was uniformly distributed from the center to the surface of all calculi (Figure 2). The matrix was composed of broad bands of parallel fibers with an amorphous interfibrillar material. These bands had a tendency to form circular whorls so that a single section usually presented some of the bands with the long axes of the fibers parallel to the plane of section and others at right angles (Figure 3). The physical characteristics of the calculus were definitely related to the structure of the matrix. Smooth surfaced calculi were found to exhibit broad bands of parallel fibers with little tendency to small whorls (Figure 3-A and C). The calculi with nodular surfaces presented small compact circular whorls of matrix

FIG. 1. RENAL CALCULI WITH CALCIFIED CENTERS AND COVERING LAYER OF UNCALCIFIED MATRIX

A. Plain roentgenogram showing two calcific densities within the right renal pelvis.

B. Retrograde pyelogram demonstrating the radiolucent uncalcified matrix.

C. Calculus sectioned to show the calcified center (a) and the laminated uncalcified matrix.

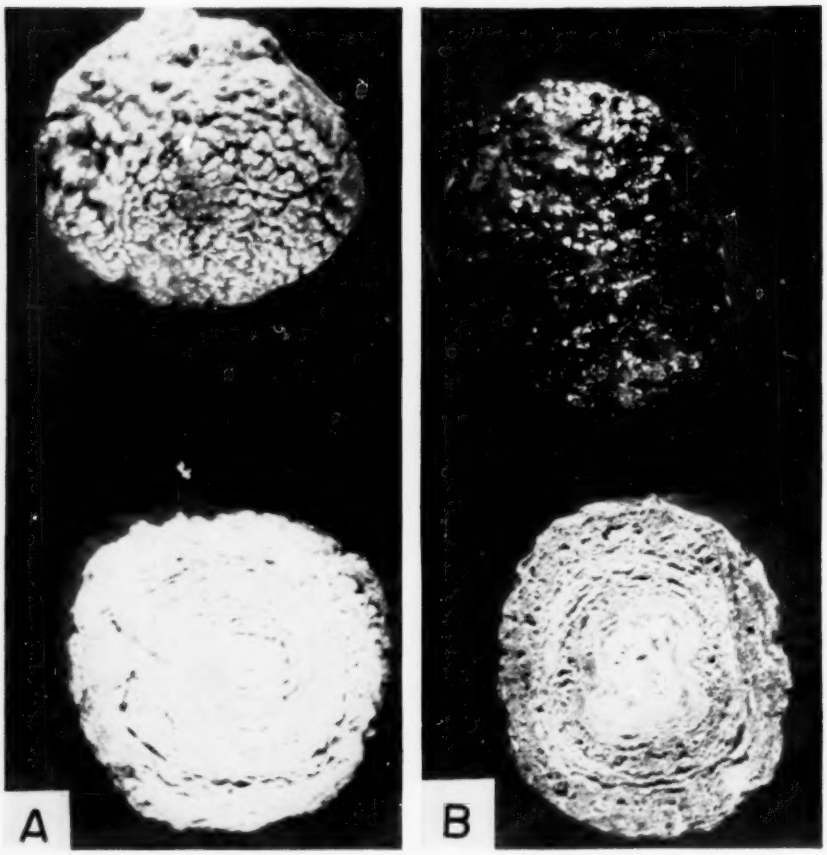

FIG. 2. MIXED CALCIUM PHOSPHATE AND OXALATE RENAL CALCULUS

A. Surface and center section of unstained calculus.

B. Surface and center section from opposite half of same calculus treated with aqueous toluidine blue. Note distribution of stained organic material throughout the calculus and on the surface. Several "nuclei" are visible near the center of the calculus.

fibers which formed a single nodule (Figure 3-B). Broad bands and small whorls of fibers were encountered at any level from center to surface of the various calculi. Sections through the central "core" of partially decalcified stones revealed only a small compact whorl of matrix and inorganic crystals (Figure 6-A). In no instance was there evidence of a nidus of cellular debris, bacteria, or other foreign material. Cross sections of many calculi revealed multiple "nuclei" (Figure 2-B) which were found to be compact whorls of organic matrix (Figure 3-B).

Between the fibrils a variable quantity of amorphous material was found in all specimens. This was most abundant in surface layers of *uncalcified* matrix in which the fibrillar structure was not as well developed as in the decalcified matrix (Figure 5). The material which diffused into the solution from the decalcifying process, and which was recovered on the collodion membranes of the ultrafilter, was composed entirely of this amorphous substance.

Cellular elements were abundant within the body of many calculi and were occasionally seen in the surface layers of the matrix (Figure 4). Segmented polymorphonuclear leukocytes, monocytes, and eosinophils were present in the inner layers of some calculi (Figure 3-A). Sheets of

lymphocytes were rarely encountered, but sometimes occurred in such numbers as to suggest a "lymphocytic stroma" in small areas of the calculus (Figure 4-A). The most uniformly present and most abundant cells encountered in the entire series were bundles of smooth muscle. These appeared to be viable cells encountered sporadically from the deepest to the most superficial layers of the calculi. These cells were often associated with fibroblasts, capillaries, and a few large cells which appeared to be transitional epithelium but occasionally resembled "foam cells". Approximately one-third of the specimens examined showed cellular elements to be present in some of the sections. Bacteria were observed in only 10 per cent of the specimens. These cellular elements were so ir-

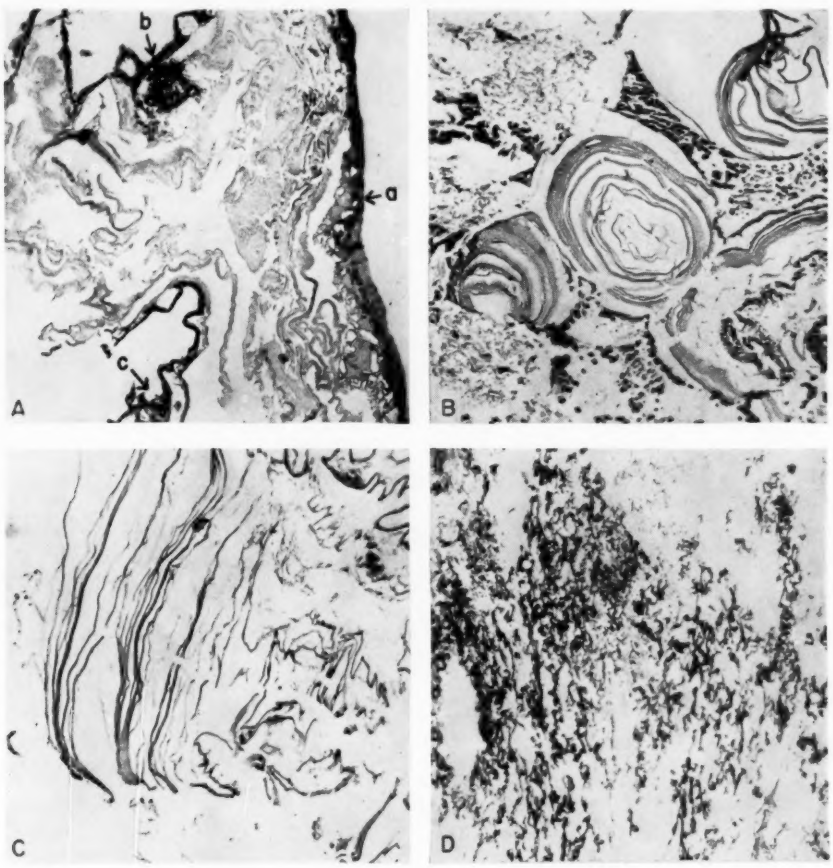

FIG. 3. PHOTOMICROGRAPHS OF DECALCIFIED RENAL CALCULI

A. Section of matrix from calcium phosphate calculus stained with hematoxylin and eosin, 10 × ocular and 10 × objective. Surface layer of matrix (a) contains a few epithelial cells, and body of calculus contains cellular elements (b and c).

B. Section from center of calculus in Figure 2, 10 × ocular and 10 × objective. Mucicarmine, iron-hematoxylin, metanil yellow treatment stains matrix red and residual inorganic crystals a deep violet.

C. Section of decalcified matrix from pure calcium phosphate calculus treated by periodic acid Schiff sulfite leucofuchsin method, 10 × ocular and 10 × objective.

D. Section of decalcified matrix from mixed calcium phosphate and oxalate calculus treated by periodic acid Schiff sulfite leucofuchsin method, 10 × ocular and 10 × objective.

regularly distributed in the various calculi, and were absent in so many of the sections, that they were considered to represent elements of granulation tissue which had extended into the interstices of the calculi and thus were probably secondary to the inflammatory reaction produced by the stone or associated infection. The well preserved structural and staining characteristics of these cellular elements indicates that the technique of decalcification, fixation, sectioning, and staining of the stone was not so severe as to disrupt the organic structure to any great extent.

Hematoxylin and eosin stain. Both the fibrils and the amorphous material of the matrix stained faintly with eosin. No histochemical importance is attached to these stains which are primarily designed to delineate the cellular elements, bacteria, and structural characteristics of the matrix.

Periodic acid Schiff and sulfite leucofuchsin reactions. The entire matrix of all calculi was colored a brilliant purplish-red with this technique (Figures 5-A and B). The fibrils appeared to stain more brilliantly and a deeper red than did the amorphous interfibrillar material, but both were strongly PAS positive. No difference in this reaction was noted between those sections sub-

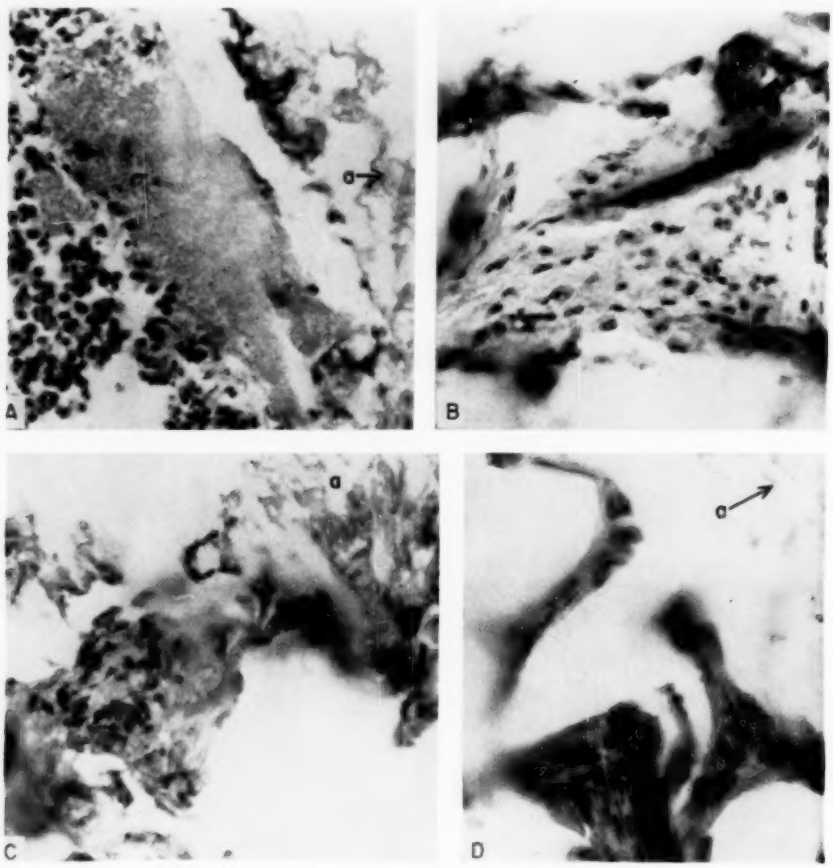

FIG. 4. CELLULAR ELEMENTS ENCOUNTERED WITHIN THE BODIES OF VARIOUS CALCULI

All sections stained with hematoxylin and eosin, 10× ocular and 44× objective. Smooth muscle, fibroblasts, lymphocytes and monocytes are visible. Transitional epithelial cells appear in some areas and unidentified "foam cells" were seen in some sections. Matrix (a) stains very faintly pink with eosin.

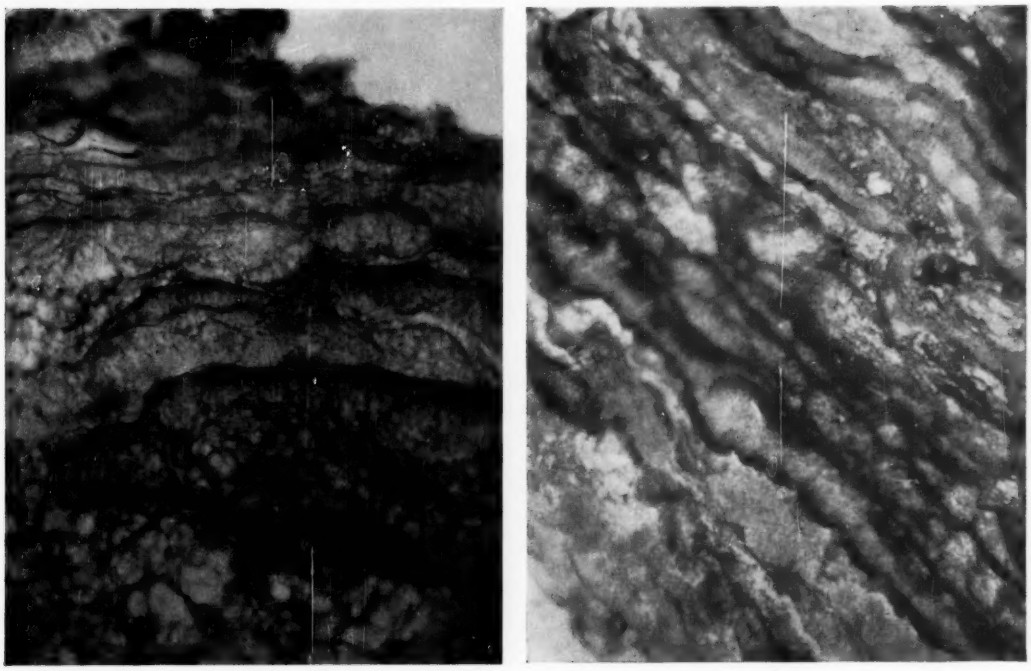

FIG. 5. METACHROMASIA IN CALCULOUS MATRIX

A. Section of uncalcified matrix from calculus in Figure 1, 10× ocular and 44× objective. Light areas (a) stain intense purplish-red, dark staining fibrils are orthochromatic with aqueous toluidine blue.

B. Section corresponding to A treated with the periodic acid Schiff sulfite leucofuchsin technique.

C. Decalcified matrix from calcium oxalate calculus stained with toluidine blue, 10× ocular and 44× objective. Most of the matrix is orthochromatic but areas of metachromasia (a) are present.

D. Decalcified matrix from calcium phosphate calculus stained with toluidine blue, 10× ocular and 44× objective. Most of the matrix is orthochromatic but areas of metachromasia (a) are present.

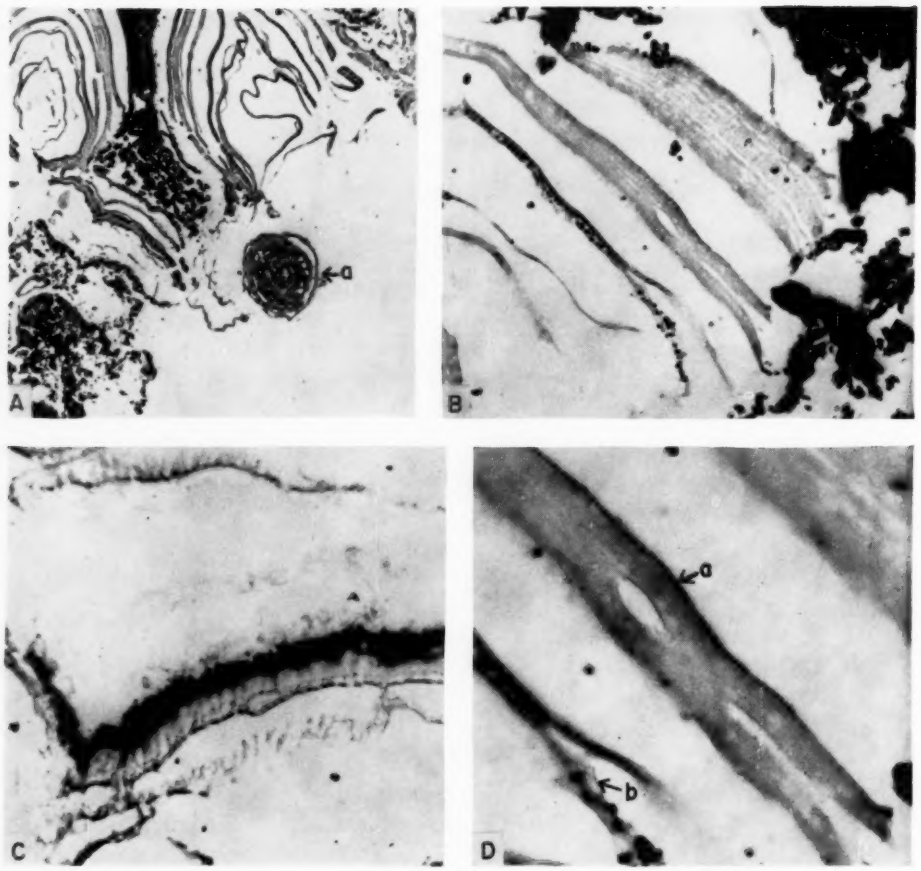

FIG. 6. DISTRIBUTION OF INORGANIC CRYSTALS IN PARTIALLY DECALCIFIED CALCULI

A. Von Kossa method with safranin counter stain through the center of mixed calcium phosphate and oxalate calculus, 10 × ocular and 10 × objective. Small spherule of matrix and crystals (a) may be the initial stage in formation of some calculi.

B. Von Kossa method with mucicarmine, iron-hematoxylin, metanil yellow counter stain of section from calcium phosphate calculus, 10 × ocular and 44 × objective.

C. Same as A, 10 × ocular and 115 × objective, to show distribution of crystals (black) along margins of fibrils.

D. Same as B, 10 × ocular and 115 × objective. Marginal silver deposits (a) may represent binding of silver ions by organic groups rather than inorganic phosphates. Amorphous material surrounding crystalline deposit is shown at (b).

jected to diastase treatment as compared with the control sections, indicating that the positive reaction is not due to the presence of glycogen. Weigert's acid iron hematoxylin counterstain obscured the PAS reaction in many areas of all sections, but there was no uptake of the hematoxylin stain by the matrix.

Toluidine blue. The matrix of all stones stained uniformly and deeply orthochromatically blue. However, there were areas of varying degree of metachromasia in every stone regardless of the crystalline composition (Figure 5). This confirms the observations reported by Howard (15) for phosphatic calculi. In our experience oxalate calculi also contain some metachromasia if the matrix is serially sectioned.

The uncalcified matrix demonstrated a very deep purplish-red metachromasia involving especially the peri-fibrillar areas (Figure 5-A). This metachromasia appeared to be enhanced by prolonged treatment of the uncalcified matrix through the decalcifying process, hence processing of the calcified stones did not destroy the metachromatic properties of the matrix.

Treatment of the matrix in concentrated sulfuric acid resulted in an increase in the metachromatic properties of the decalcified matrix. This was attributed to sulfation of the PAS positive mucoprotein (5) rather than to the removal of additional cationic groups since a reducing acid rinse was utilized prior to the PAS treatment of control sections.

von Kossa stain for "calcium salts." This test depends upon the presence of insoluble substances which can bind silver ions in such a manner that after exposure to sunlight they can subsequently be reduced by developer and the unbound silver cleared with thiosulfate in the same manner as photographic emulsions. The insoluble phosphates, alkali salts of fatty acids, and free aldehyde groups of large molecules may give positive results with the stain. The large crystalline deposits (Figure 6) which give the von Kossa stain are considered to be calcium phosphate crystals because the inorganic ash of these preparations is composed almost entirely of calcium phosphate, and because the same deposits stain a deep blue with alum hematoxylin and grey-violet with iron hematoxylin in corresponding sections. The thin line of reduced silver along the margins of the fibrils (Figure 6-D) may not be phosphate crystals, but may represent free aldehyde or other groupings capable of binding silver ions.

The black deposits of "calcium salts" were found to occur in linear arrangements along the borders of the fibrillar matrix and appeared to be intermingled with the amorphous interfibrillar portions of the matrix (Figure 6-C).

Stains for mucin. Treatment with the mucicarmine, iron hematoxylin, metanil yellow stain of Mayer resulted in a diffuse red color in the matrix. It was noted that incompletely decalcified matrix demonstrated a tendency to stain yellow along the borders of fibrils where calcium salts were deposited (Figure 6-D). Mucihematein stained the entire matrix a diffuse light violet.

Tests for lipids. No specimen demonstrated any indication of lipid soluble staining with Sudan black. This was interpreted as evidence that the PAS positive substance was not partially oxidized lipid such as lipofuscin or "ceroid." In addition to these studies, which were made on sections from all specimens, the lyophilized matrix from pooled calculi was tested for the presence of lipids with osmic acid and by the Wilson-Hanner method for determination of serum lipids (16). No evidence of free or protein bound lipids could be detected in any of the specimens.

Chemical studies of stone matrix

The quantity of matrix recovered from the various types of crystalline calculi is presented (Table I). The matrix, as recovered from the decalcifying solution, contained 5.22 to 14.98 per cent of inorganic ash as determined by ignition of the material in the presence of sulphuric acid. The cationic component of the ash was calcium with only traces of magnesium. The major anionic component was phosphate. The ash is expressed therefore as largely calcium phosphate with the excess of calcium as the sulfate. The average quantity of ash-free matrix in all calculi was 2.52 gm. per 100 gm. of dry calculus.

It was observed that between 40 and 60 per cent of the lyophilized stone matrix, which had not been fixed in formalin, was soluble in molar sodium chloride by repeated extractions. The soluble and insoluble forms of the matrix were recovered by relyophilization and subjected to the same studies as the total matrix. Microscopic sections revealed the soluble material to be composed entirely of the amorphous portions of the matrix, and the insoluble material to be composed largely of the fibrillar material. These two fractions of the matrix revealed entirely similar histochemical reactions to all of the above tests with the exception that metachromasia with toluidine blue was occasionally demonstrated in the soluble amorphous material but not in the fibrillar residue.

It was observed that the soluble portion of the stone matrix was electrophoretically homogeneous and retained a net negative charge (migrated toward the anode) at pH 4.5, 8.6, and 12.0. The electrophoretic mobility at pH 8.6 in veronal buffer of ionic strength 0.1 was -5.61×10^{-5} cm.^{2}

TABLE I

Amount of lyophilized matrix recovered from calcigerous urinary calculi

Type of calculi	Weight			Corrected amount of	
	Dry stones (*gm.*)	Matrix (*gm.*)	Per cent	Inorganic ash in matrix (*per cent*)	Matrix in stones (*per cent*)
Pool No. 1, 100 stones—mixed Ca-oxalate and Ca-phosphate	68.45	2.2041	3.22	12.46	2.82
Pool No. 2, 50 stones—Calcium phosphate	40.24	1.0001	2.48	6.84	2.32
Pool No. 3, 25 stones—Ca-phosphate and Mg-NH_4 phosphate	35.84	0.7526	2.10	5.22	2.00
Pool No. 4, 88 stones—Calcium oxalate	35.19	1.3358	3.79	14.98	3.22
Pool No. 5, single calculus, Ca-phosphate and Mg-NH_4 phosphate	25.23	0.5940	2.35	5.37	2.23
Total	204.95	5.8866	2.79	8.97	2.52

volt per sec. under a potential gradient of 6.6 volt per cm. for a duration of 7,200 sec.

Elemental analyses of the matrix. Table II is a compilation of the average elemental composition of matrix recovered from five pools of calculi. The analysis of the total matrix is compared with that of the molar sodium chloride soluble and insoluble fractions of the same pools. These results have been corrected for inorganic ash content.

Spectroscopic analysis of the ash revealed calcium and phosphorus to be the predominant elements, although traces of magnesium were usually present. The inorganic ash from lyophilized matrix was analyzed for calcium content. The amount of calcium in the matrix from the 5 pools of calculi varied between 0.56 and 4.49 per cent (wt/wt). The amount of calcium in the ash from lyophilized matrix obtained from 5 pools of calculi exceeded the amount of $Ca_3(PO_4)_2$ as calculated from the total phosphorus present in every sample. This "excess" calcium accounted for 0.19 to 0.64 per cent of the total matrix.

Tests for protein. The biuret, ninhydrin, xanthoproteic and Millon tests were all positive for both the sodium chloride soluble and insoluble portions of the matrices. When human serum albumin was utilized as a standard the biuret test indicated a protein content of 68 to 73 per cent for 5 determinations of total matrix. The ninhydrin reaction was much more pronounced after hydrolysis, indicating an increase in free amino groups and suggesting the possibility that hexosamine may be present as N-acetyl-hexosamine.

General tests for carbohydrates in total matrix. The anthrone test produced a blue color with absorption maximum at 625 mμ. The Molisch test also gave a positive reaction with peak absorption at 570 mμ. The orcinol test gave a brown to yellow color with absorption maxima at 420 and 540 mμ, indicating the presence of hexoses other than hexosamine. The hexose content of total matrix from 5 pools of calculi varied between 10.6 and 15.3 per cent with a mean value of 13.6 per cent. The Di Ferrante test was faintly positive, but this method for the detection of acid polysaccharides has been reported to be of limited accuracy in the presence of proteins (9). The naphthoresorcinol test for hexuronic acids was

TABLE II

Elemental analysis of stone matrix

Mean results from five pools of calculi (± S.D.); corrected for inorganic ash content

	Carbon	Hydrogen	Nitrogen	Conjugated sulfate sulphur
Total matrix	57.24 (±1.22)	7.03 (±0.13)	10.32 (±0.53)	0.98 (±0.29)
NaCl soluble portion	58.62 (±0.81)	7.40 (±0.84)	10.65 (±0.83)	0.78 (±0.17)
NaCl insoluble residue	56.80 (±3.61)	7.21 (±0.46)	10.32 (±0.48)	0.78 (±0.26)

negative, but this test has been reported unsatisfactory for the determination of hexuronic acids in acid mucopolysaccharides (17). The carbazole reaction of Dische also gave a negative test for hexuronic acids. The sensitivity of this test was such that 0.05 to 0.1 per cent hexuronic acid content of the matrix would have been detected. The diphenylamine test for "sialic acid" as described by Winzler (7) and also by Anderson and Maclagan (8) was negative for stone matrix, although control studies on Tamm and Horsfall mucoprotein (18) and on urinary mucoprotein isolated by the Anderson-Maclagan technique were strongly positive.

Both the Elson-Morgan (7) and the Dische-Borenfreund (19) reactions gave a positive test for hexosamine. The hexosamine content of total matrix from 5 pools of freshly recovered calculi averaged 14.0 per cent (range 11.8 to 16 per cent) by the Elson-Morgan reaction. The Dische-Borenfreund reaction gave an average hexosamine content of 14.6 per cent for the same preparations. The agreement between these two methods is a further indication that neither hyaluronic nor chondroitin-sulfuric acids are present since these acid mucopolysaccharides produce marked discrepancies in the results obtained by the indole-HCl technique as compared with the Elson-Morgan reaction.

Chromatographic study of carbohydrate and amino acid content. A 16-mg. sample of total matrix was hydrolyzed with 10 ml. of 6 N HCl for 20 hours in a sealed tube at 100° C. Two-dimensional chromatograms with phenol and lutidine solvents were made on Whatman No. 1 filter paper according to the method described by Block, Durrum, and Zweig (20). The amino acids threonine and leucine (isoleucine) were present in largest concentration. Serine, glycine, tyrosine, and hexosamine were also identified. Arginine was thought to be present but was poorly separated from lysine, and two materials which stained poorly with ninhydrin could not be identified.

A 100-mg. sample of the total martix was hydrolyzed with 5 ml. of N H_2SO_4 at 100 C. for 6 hours in a sealed vial. The hydrolysate was neutralized with $BaCO_3$ and the supernatant subjected to two-dimensional chromatography utilizing l-butanol : acetic acid : water (4 : 1 : 5, v/v) and l-propanol : ethyl acetate : water (7 : 1 : 2, v/v) as solvents. The chromatogram was developed with 1 per cent alcoholic resorcinol in an equal volume of 0.2 N HCl. Galactose was definitely indicated as the predominant sugar but mannose was not detectable. One other pink staining material was present but could not be identified. It was determined not to be glucuronic acid, galacturonic acid, glucose, fructose, arabinose, maltose, lactose, raffinose or xylose.

DISCUSSION

Histochemically the calculus matrix is composed of a "neutral mucoprotein" which gives the positive PAS reaction and a "sulfated mucopolysaccharide" which is metachromatic with toluidine blue. Microscopically no distinct structural separation of the two substances has been demonstrable. Since the amount of metachromatic material varied considerably from one calculus to another, one would anticipate variations in the carbohydrate content of calculi in proportion to the variations in relative concentration of muco polysaccharide to mucoprotein. There has been little variation in the quantity of protein-bound hexose and hexosamine in 5 pools of freshly recovered matrix from calculi obtained from patients who were free of infection. Studies now being prepared for publication have demonstrated a wide range of variation in the total hexose plus hexosamine content of calculi which were permitted to dry at room temperature prior to decalcification, or which were recovered from patients with urinary tract infection. However, the quantity of hexose to hexosamine has remained at very near 1 : 1 ratio, or equimolar concentration, throughout the range of these variations in total carbohydrate content.

These preliminary studies offer insufficient evidence for conclusions as to the source of origin of the matrix. A number of carbohydrate-protein substances are present in normal and pathologic urine. Tamm and Horsfall (18) were the first to isolate and characterize the urinary mucoprotein which inhibits hemagglutination by certain viruses. The urinary mucoproteins recovered by Anderson, Lockey, and Maclagan (21) are in part composed of T & H mucoprotein. Blood group substances of types A, B, and O (22) have been recovered from normal urine. Acidic mucopoly-

saccharides which exhibit metachromasia, contain hexuronic acid, and have electrophoretic and chromatographic properties similar to chondroitin sulphate have been isolated from normal urine (23). Heparin may be a constituent of normal urine (24). Serum glycoproteins (MP-2) are present in relatively large concentration in normal urine (25), and orosomucoid (MP-1) has been demonstrated in the urine of children with the nephrotic syndrome by Popenoe (26). Hamerman, Reife, and Bartz (27) have demonstrated a correlation between the serum and urinary levels of protein-bound hexosamine and hexose in several diseases. The gonadotropic hormones are also mucoproteins which occur in the urine (28). Many bacteria and fungi produce polysaccarides containing aminosugars (29), and these substances are undoubtedly present in the urine of patients with pyelonephritis due to *Proteus vulgaris, B. coli, Staphylococcus aureus,* and other polysaccharide producing organisms.

None of the above mucosubstances appear to be closely related to the material recovered from urinary calculi. Meyer, Linker, Davidson, and Weissmann (30) have found the major polysaccharide component of bovine cornea to be a sulphated polysaccharide which does not contain uronic acid, and for which they have proposed the name keratosulfate. It is composed of equimolar quantities of glucosamine, acetyl, galactose, and sulfate. Two somewhat similar acidic substances which are metachromatic and which contain no uronic acids have been found in rabbit and human connective tissues by Consden and Bird (31). If one permits allowances for some loss of ester sulphate, then a polysaccharide of the above composition conjugated with a protein moiety in the ratio of approximately 34 per cent polysaccharide to 66 per cent protein would result in a molecule very similar to that of stone matrix. The future characterization of these mucosubstances must inevitably lead to the identification and the source of origin of the organic matrix of urinary calculi.

SUMMARY

The organic matrix is evenly distributed from the center to the surface of all calcigerous urinary calculi, and quantitatively comprises approximately 2.5 per cent of the weight of each calculus. Microscopically the matrix appears as a mixture of amorphous and fibrillar forms which are closely related in elemental composition. However, wide variations in carbohydrate and amino acid content may occur within the framework of this elemental composition and the present studies offer no conclusive evidence that the amorphous and fibrillar forms of the matrix are identical substances. Histochemically matrix is composed of a combination of mucoprotein (PAS positive) and mucopolysaccharide (metachromatic), which have not been separated or identified by preliminary chemical analyses.

Galactose and hexosamine in approximately equimolar concentration form the major components of the carbohydrate moiety of stone matrix. This accounts for approximately one-third of the matrix material. If all conjugated sulphate sulfur is present in the carbohydrate moiety then this portion of the matrix contains between 9 and 14 per cent sulphate. The presence of metachromasia is considered to indicate that a part or all of this sulphate may exist in the ester form.

The protein or peptide portion of the molecule contains threonine, leucine, serine, glycine, lysine, arginine, and tyrosine with other components that have not been identified. The protein moiety accounts for approximately two-thirds of the matrix.

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STUDIES OF THE EFFECTS OF LARGE DOSES OF BACTERIAL PYROGEN IN THE DOG. I. THE RENAL HANDLING OF SALT AND WATER [1, 2]

By J. LEONARD BRANDT, BARNETT ZUMOFF,[3] LEONARD CASTLEMAN, HERMAN D. RUSKIN, AUDREY JONES, AND SEYMOUR ZUCKERMAN
WITH THE TECHNICAL ASSISTANCE OF MARY BIANCHI

(From the Department of Medicine, State University of New York, College of Medicine at New York City, Brooklyn, N. Y.)

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The augmenting effect of pyrogens on urine flow in the dog has been demonstrated and shown to be associated with increased solute excretion (1) and unresponsiveness of the renal tubule to antidiuretic doses of Pitressin® (2). The possible experimental and clinical usefulness of a group of substances which are able to increase solute excretion and interfere with antidiuretic activity is obvious.

The present report records our observations of the renal effects of pyrogen on mongrel dogs and an analysis of the mechanism of a pyrogen diuresis.

MATERIAL AND METHODS

A total of 21 female mongrel dogs were used in these experiments. All animals were studied in the postabsorptive state under light anesthesia. Initial anesthesia was produced by the intravenous injection of 25 mgm. Pentobarbital per kilogram of body weight approximately 45 minutes before the start of any experiment. Light anesthesia was maintained throughout the remainder of the experiment by the administration of 25-mgm. doses of Pentobarbital at approximately hourly intervals.

The constant infusion technique was used for the measurement of renal dynamics. Clearances of creatinine (C_{Cr}) and para-aminohippurate (C_{PAH}) were used as measures of glomerular filtration rate and renal plasma flow, respectively. Timed urine collections were made through an indwelling bladder catheter. Mid-period blood sampling was done through a polyethylene catheter from either the femoral or iliac vein. Plasma and urine osmolarity and urine flow were measured, and from these total solute excretion, free water clearance, and osmolar clearance were calculated. Timed urine collections were made for at least 45 minutes before and for more than two hours following the intravenous injection of pyrogen in all experiments.[4]

Creatinine concentrations in plasma and urine were determined by the Jaffé reaction (3); PAH concentrations in plasma and urine by the method of Smith, Finkelstein, Aliminosa, Crawford, and Graber (4); glucose concentrations by the method of Nelson (5); and chlorides by the method of Schales and Schales (6). Sodium and potassium concentrations were measured with a flame photometer using a lithium internal standard. For the determination of plasma and urine osmolarity, freezing point depressions were measured by means of a Fiske Osmometer.

The animals were divided into four groups as follows:

Group I—six dogs allowed water freely before starting the study received 30 to 50 micrograms of pyrogen [5] intravenously after a set of control periods. The sustaining infusion of test materials was made up in physiological saline, and administered at the constant rate of 2.0 cc. per minute.

Group II—six dogs in which tubular maxima for glucose (three dogs) and PAH (three dogs) were measured before and after a 30 to 50-microgram dose of pyrogen was given intravenously. The usual procedures for Tm measurements were used (7).

Group III—five dogs in which a constant intravenous water (5 per cent glucose in water at a rate of 6 cc. per minute) load was given throughout the experiment. After water diuresis was established, Pitressin® in antidiuretic doses was added to the constant infusion (25 to 50 mUnits Pitressin® per hour). After antidiuresis had been produced, the dogs received 30 to 50 micrograms of pyrogen intravenously.

Group IV—four hydropenic dogs were given large solute loads (25 per cent mannitol or 12 per cent sodium sulfate) before and after a 30 to 50-microgram dose of

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[2] A portion of this work was presented at the Seventh Annual Conference on the Nephrotic Syndrome, Boston, October 22, 1955.

[3] Present address: Sloan-Kettering Institute, New York.

[4] The appearance of what is subsequently termed "pyrogen effect" was determined by the sudden rise in urine flow during each experiment.

[5] The pyrogen used in this study was Piromen, a polysaccharide derived from the *Pseudomonas aeroginosa*. The material was supplied by Travenol Laboratories, Morton Grove, Ill., and contains ten micrograms of pyrogen per cc.

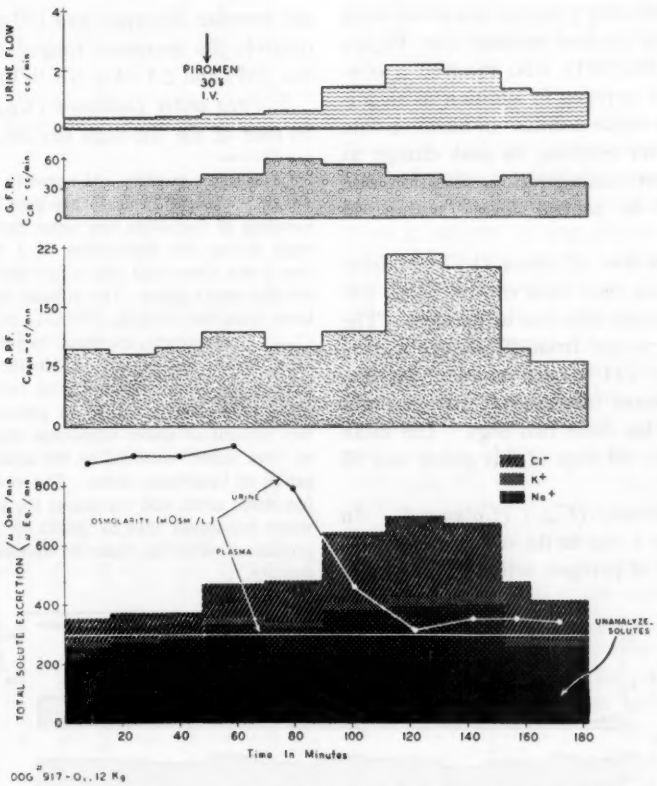

FIG. 1A. THE EFFECT OF A SINGLE DOSE OF PYROGEN ON RENAL HEMODYNAMICS, SOLUTE EXCRETION AND URINARY OSMOLARITY

Note that the maximal change in filtration rate precedes the other changes.

pyrogen. These dogs were made hydropenic by withholding water for 18 to 20 hours. One animal received an increasing load of 25 per cent mannitol and then the pyrogen; when the "pyrogenic effect" became manifest, the infusion rate was slowed so as to decrease the rate of solute loading. In the other three dogs, a constant infusion (12 per cent sodium sulfate in one and 25 per cent mannitol in two) was maintained before and after the pyrogen administration. Plasma osmolarity was measured for each collection period in each of the four dogs.

RESULTS

Group I Dogs—Table I (see also Figures 1a and 1b)

The renal plasma flow rose to a maximum about 65 minutes, on the average, after the intravenous administration of pyrogen. This was followed in about 20 minutes by the maximal changes in urine flow, total solute, sodium chloride excretion and osmolar clearance. Twenty minutes later maximal changes in filtration rate, dilution of urine, and potassium excretion occurred.

1. *Urine flow (Column 1).* In all animals there was a rise in urine flow starting approximately 50 minutes after the injection of pyrogen, the increase varying from three to eleven times that of control flow with an average increase of sevenfold. In general, maximum urine flow was reached within 30 minutes of the gradual onset of diuresis; it then decreased slightly and remained fairly constant during the rest of the study.

2. *Glomerular filtration rate (Column 2).* Of the six animals, three showed a slight increase[6]

[6] Arbitrarily, a rise of 1 to 9 cc. was considered a slight increase, 10 to 19 cc. a moderate increase, and 20 cc. or more a marked increase in filtration rate.

in filtration rate following pyrogen, one a moderate increase, and one a marked increase (see Figure 1a). One dog (No. 921) who received a continuous infusion of pyrogen in addition to an initial dose showed a slight decrease in filtration rate (Figure 1b). After reaching its peak change as a result of pyrogen administration, filtration rate usually returned to control levels within 45 minutes.

3. *Renal plasma flow* (*Column 3*). A uniform rise in renal plasma flow over control levels followed the intravenous injection of pyrogen. The average increase ranged from 49 cc. per minute (dog No. 915) to 224 cc. per minute (dog No. 925) which represent increases of 106 per cent and 125 per cent for these two dogs. The mean increase in flow for all dogs of this group was 98 per cent.

4. *Osmolar clearance* (C_{osm}) (*Column 4*). In all dogs there was a rise in the osmolar clearance during the period of pyrogen activity. The average osmolar clearance was 150 per cent above the control; the increases ranged between 0.6 (dog No. 955) and 2.3 (dog No. 921) cc. per minute.

5. *Free water clearance* (C_{H_2O}[7]) (*Column 5*). In four of the six dogs studied, free water clear-

[7] The terms positive and negative free water clearance (+ or $-C_{H_2O}$ in Table I) are used to describe the renal handling of the solute free water moiety in hydrated animals during the elaboration of a hypertonic (negative free water clearance) and a hypotonic (positive free water clearance) urine. The volume of free water is calculated from the formula, $V = C_{osm} + C_{H_2O}$, or $C_{H_2O} = V - C_{osm}$. Conceptually, the term "negative free water clearance" means the amount of "distilled" water one would have to add to one minute's urine excretion to restore that urine to isotonicity (equal to plasma osmolarity); thus, this amount of water represents the per minute volume of "free water" removed by the tubules during the elaboration of hypertonic urine. The reverse is applicable to hypotonic urine, and represents the per minute volume of water one would have to "distill off" from a minute's excretion of urine in order to restore that urine to isotonicity.

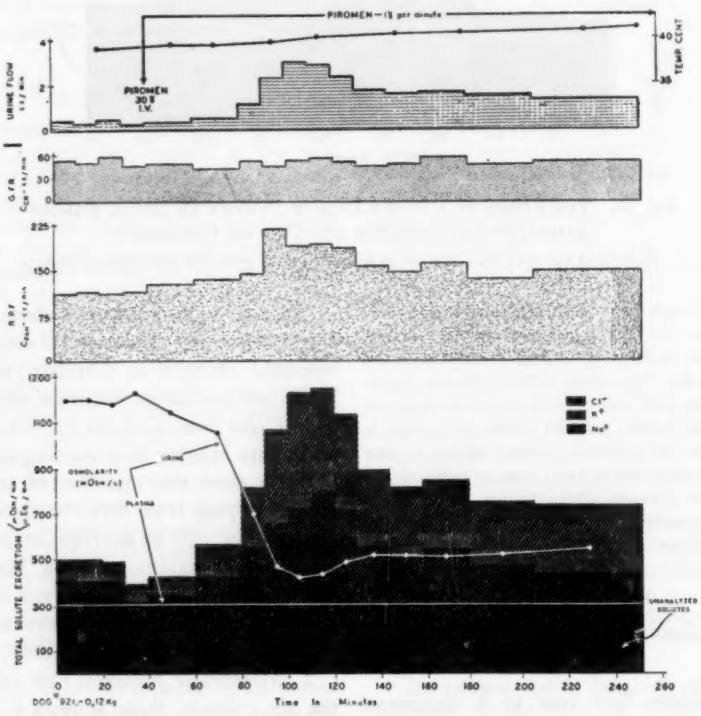

FIG. 1B. THE EFFECT OF A SINGLE DOSE PLUS A CONTINUOUS INFUSION OF PYROGEN ON RENAL HEMODYNAMICS, SOLUTE EXCRETION AND URINARY OSMOLARITY

Note that there is no significant change in filtration rate.

TABLE I

Composite data of Group I dogs *

Dog	Urine flow cc./min. (1)	GFR-C_{In} cc./min. (2)	RPF-C_{PAH} cc./min. (3)	C_{osm} cc./min. (4)	C_{H_2O} cc./min. (5)	U_{osm} mOsm./L. (6)	OSM U/P (7)	Per minute excretion rates				
								Solutes μOsm. (8)	Sodium μEquiv. (9)	Potassium μEquiv. (10)	Chloride μEquiv. (11)	
915	0.4	42	46	1.4	−1.0	1,160	3.9	400	14	7	101	
	3.7(5.0)	47(55)	95(119)	3.2(4.0)	0.5(1.0)	263(233)	0.9	964(1,200)	403(510)	33(47)	286(424)	
917	0.5	36	94	1.2	−0.8	895	3.0	363	45	14	79	
	1.9(2.2)	44(60)	177(216)	2.2(2.3)	−0.4(−0.1)	373(315)	1.2	669(694)	179(197)	31(37)	250(268)	
925	0.55	82	179	1.2	−0.65	658	2.2	366	72	13	127	
	3.2(4.1)	99(125)	403(460)	3.1(3.7)	0.04(1.9)	300(254)	1.0	974(1,121)	288(327)	57(70)	337(408)	
921†	0.4	52	119	1.6	−1.2	1,225	4.1	462	55	16	59	
	2.7(3.0)	49(53)	196(213)	3.9(4.2)	−1.2(−1.1)	441(415)	1.5	1,178(1,246)	390(428)	46(54)	390(454)	
921A	0.2	41	67	0.8	−0.6	957	3.2	253	14	11	34	
	2.2(2.4)	61(67)	139(152)	2.7(2.8)	−0.5(−0.35)	377(344)	1.25	816(854)	219(238)	44(54)	246(254)	
955	0.4	44		1.1	−0.7	869	2.9	323	10	3	29	
	1.2(1.9)	52(80)		1.7(2.1)	−0.5(−0.15)	589(465)	2.0	513(637)	103(147)	12(20)	162(249)	

* The upper row of figures for each dog is the mean of three control collection periods; the lower being the mean of three consecutive periods during the pyrogen effect. Data in parentheses are the maximum changes during the pyrogen effect.

† This dog received one microgram of pyrogen per minute in addition to a single 30-microgram dose.

ance became less negative or even positive following the administration of pyrogen. In one dog (No. 921) the free water clearance remained unchanged.

6. *Urine osmolarity* (*Column 6*). In all dogs there was a fall in urine osmolarity, during the period of pyrogen activity, towards that of the plasma. The mean and standard deviation of the osmotic activity of dog plasma in this series is 310 ± 14 mOsm. per L. From an average control level of 994 mOsm. per L. urine osmolarity fell to an average of 401 mOsm. per L. at the height of pyrogen activity.

7. *Osmotic U/P ratio* (*Column 7*). In all dogs there was a fall in the osmotic U/P ratio toward 1.0. The highest control osmotic U/P ratio was 4.1 which fell to 1.5 during pyrogen diuresis (dog No. 921). The smallest fall in osmotic U/P ratio was seen in dog No. 955 (2.9 to 2.0).

8. *Excretion of solutes.*

A. *Total solutes* (*Column 8*). There was a uniform rise in total solute excretion for all dogs during the period of pyrogen activity as compared with the rates of solute excretion during the control period. The increase in solute excretion ranged between 190 μOsm. per minute (dog No. 955) and 716 μOsm. per minute (dog No. 921). Inspection of Table I and Figures 1a and 1b show that the increased excretion of sodium, chloride, and potassium accounts in the main for the increase in total solute excretion, and there is no gross change in the excretion of other urinary solutes.

B. *Sodium and chloride excretion* (*Columns 9 and 11*). These two ions showed the most striking increase in excretion rate during pyrogen activity. The smallest change in the rate of sodium excretion was a tenfold increase from 10 μEq. per minute to 103 μEq. per minute (dog No. 955). The greatest change was a thirtyfold increase from 14 μEq. to 403 μEq. per minute at the height of the pyrogenic effect (dog No. 915). Chloride excretion on the other hand ranged from a threefold to a sevenfold increase in this group of animals.

C. *Potassium excretion* (*Column 10*). This ion showed a large increase in excretion rate during the pyrogenic effect, although it was not as marked as the effects on sodium and chloride.

TABLE II

Effect of pyrogen on Tm_G and Tm_{PAH}

Dog No.	Control *mgm./min.*	Pyrogen diuresis *mgm./min.*
	A. Tm_{PAH}	
169	28.1	19.2
285	15.1	17.0
182	12.5	17.6
	B. Tm_G	
169	207	208
265	177	298
115	127	142

Potassium excretion increased fourfold except in dog No. 917 where only a twofold increase from 14 μEq. per minute to 31 μEq. per minute was noted.

Items 6 to 8 A-C indicate that more solute and especially more electrolyte is excreted during the period when pyrogen acts on the kidney, at the same time that more free water is excreted.

Group II dogs—Table II

In the three dogs in whom the tubular maxima for PAH (Tm_{PAH}) were studied during control periods and during a "pyrogenic effect," one showed a decrease, one remained essentially unchanged, and in the third there was a rise.

The same variability in response was noted in the three dogs in whom the tubular maxima for glucose (Tm_G) were measured before and after the administration of a dose of pyrogen. One dog showed a fall following pyrogen, another a rise, and the third only a slight change.

Group III dogs—Table III

For the five animals in this group the lowest urine osmolarity during the initial water diuresis ranged between 66 mOsm. per L. and 145 mOsm. per L. and averaged 111 mOsm. per L. During the antidiuresis resulting from the Pitressin® infusion, urine osmolarity rose to peaks ranging between 615 mOsm. per L. and 1400 mOsm. per L. and averaged 944 mOsm. per L. During the period of pyrogen activity, despite the continued Pitressin® infusion, urine osmolarities fell to between 60 mOsm. per L. and 116 mOsm. per L., the average being 93 mOsm. per L.

During the period of Pitressin® antidiuresis osmolar clearances remained essentially unchanged (a mean of 1.5 cc. per minute from a mean of 1.6 cc. per minute during initial water loading). There was associated a marked and precipitous fall in free water clearance during Pitressin® antidiuresis (a mean of minus 0.9 cc. per minute from a mean of plus 2.7 cc. per minute during the initial water loading). The antidiuretic effect was reflected in the fall in urine flow from 4.3 cc. per minute during water loading to 0.6 cc. per minute. With the onset of inhibition of the Pitressin® antidiuresis by pyrogen, osmolar clearances remained constant, but the free water clearance rose to a higher mean level (plus 3.4 cc. per minute) than was noted during the initial phase of water loading. Concomitantly, the mean urine flow reached higher levels (4.9 cc. per minute) than those attained during the same initial period.

Group IV dogs—Table IV, Figure 2

For the three dogs in whom the rate of solute loading was kept constant, urine flows averaged 3.9, 4.8, and 5.1 cc. per minute; total solute excretions averaged 2.46, 2.75, and 3.0 mOsm. per minute, respectively, during control periods. For the same periods osmolar clearance and $T^{Cl^-}_{H_2O}$[8]

[8] The symbol $T^{C}_{H_2O}$ (8) will be used to designate the removal of solute free water during a sustained concen-

TABLE III

The effect of pyrogen in water loaded dogs in which the water diuresis was inhibited by Pitressin®

Dog No.	Initial water load				Pitressin® effect				Pyrogen effect			
	Min. U_{osm} *mOsm./L.*	Urine flow *cc./min.*	C_{osm} *cc./min.*	C_{H_2O} *cc./min.*	Max. U_{osm} *mOsm./L.*	Urine flow *cc./min.*	C_{osm} *cc./min.*	C_{H_2O} *cc./min.*	Min. U_{osm} *mOsm./L.*	Urine flow *cc./min.*	C_{osm} *cc./min.*	C_{H_2O} *cc./min.*
27	100	3.6	1.2	+2.4	1,100	0.3	1.2	−0.9	104	4.0	1.4	+2.6
53	126	4.0	1.7	+2.3	835	0.6	1.6	−1.0	116	4.4	1.7	+2.7
52	120	7.0	2.8	+4.2	615	1.5	3.0	−1.5	107	6.9	2.5	+4.4
82	66	4.7	1.0	+3.7	770	0.3	0.7	−0.4	60	6.5	1.3	+5.2
96	145	2.2	1.1	+1.1	1,400	0.2	0.9	−0.7	79	2.8	0.7	+2.1
Mean	111	4.3	1.6	+2.7	944	0.6	1.5	−0.9	93	4.9	1.5	+3.4

TABLE IV

Data from hydropenic, solute-loaded dogs given pyrogen *

Urine flow *cc./min.*	Tot. solute excr. *mOsm./min.*	C_{osm} *cc./min.*	$T^{C}_{H_2O}$ *cc./min.*	Period
Dog No. 414—Constant Infusion—25% Mannitol				
5.0	2.91	8.8	3.8	Control
5.6	3.28	9.9	4.3	Control
4.8	2.84	8.6	3.8	Control
5.8	2.72	8.2	2.4	Pyrogen effect
6.3	2.74	8.2	1.9	Pyrogen effect
5.7	2.66	7.8	2.1	Pyrogen effect
Dog No. 285—Constant Infusion—25% Mannitol				
3.9	2.46	7.4	3.5	Control
4.0	2.48	7.4	3.4	Control
3.9	2.45	7.3	3.4	Control
5.0	2.15	6.4	1.4	Pyrogen effect
5.3	2.22	6.6	1.3	Pyrogen effect
5.3	2.28	6.7	1.4	Pyrogen effect
Dog No. 416—Constant Infusion—12% Sodium Sulfate				
4.8	2.69	8.0	3.2	Control
4.8	2.79	8.3	3.5	Control
4.9	2.81	8.4	3.5	Control
6.3	2.72	7.9	1.6	Pyrogen effect
6.3	2.77	7.9	1.6	Pyrogen effect
5.7	2.70	7.9	1.2	Pyrogen effect
Dog No. 415—Changing Infusion Rate—25% Mannitol				
5.9	3.3	9.0	3.1	Control
8.8	4.6	13.0	4.2	Control
11.5	6.0	16.2	4.7	Control
12.5	6.6	17.3	4.8	Control
15.6	8.0	20.3	4.7	Control
16.9	8.6	21.7	4.8	Control
16.0	7.9	20.0	4.0	Pyrogen effect
13.8	6.8	17.0	3.2	Pyrogen effect
13.0	6.3	15.7	2.7	Pyrogen effect
10.8	5.2	12.9	2.1	Pyrogen effect
8.5	4.3	10.6	2.1	Pyrogen effect

* See text for details.

averaged 7.35, 8.20, and 9.0 cc. per minute and 3.4, 3.4, and 4.0 cc. per minute, respectively.

During the "pyrogenic effect" urine flow rose in all animals, and there was a slight decrease in solute excretion. Associated with the rise in urine flow there was a fall in urine osmolarity and a decrease in $T^{C}_{H_2O}$.

Figure 2 is a plot of osmolar clearances against urine flows for an animal in whom the rate of infusion of solute was varied. It can be seen that during control periods, before the administration of pyrogen, the tubular removal of solute free water by the concentrating apparatus reaches a maximum ($Tm^{C}_{H_2O}$) of 4.8 cc. per minute at urine flows above 9.0 cc. per minute (line A). During the "pyrogenic effect," this maximal rate of free water removal is decreased by more than half (line B).

DISCUSSION

The complex interrelationship between filtration rate and hormonal action on glomerular and tubular factors in determining the quality and quantity of elaborated urine is well known. No consistent relationship exists between renal plasma flow and the quality and quantity of excreted urine. The present studies have demonstrated that in response to intravenous pyrogen in proper doses normally hydrated anesthetized animals receiving a saline infusion develop a striking rise in solute excretion made up chiefly of sodium, potassium, and chloride. The consistent rise in solute excretion is usually, but not invariably, accompanied by a rise in filtration rate (see Figures 1a and 1b). The effects of a saline infusion on urine flow and solute excretion in dogs who do not receive pyrogen are minimal as compared to those receiving pyrogen over a similar period of time (11). Since there is no consistent alteration of proximal function (Tm_G measurements in dogs of Group II), the changes can scarcely be ascribed to tubular damage of a non-specific type. While physiological alterations of proximal tubular handling of solute and water may be induced by a large dose of pyrogen, and should not be ignored, their magnitude (if any) cannot be determined by ordinarily available methods. The exact mechanism of the increased electrolyte excretion seen during the pyrogenic effect is not clear. The excretion of electrolyte usually rises to a peak coincident with or shortly after the rise in filtration rate (see Figure 1b), declines slightly and is then maintained at a high level for many hours while the filtration rate returns to pre-pyrogen levels. This suggests a transient glomerular component

trating stimulus (hydropenia plus solute loading). This symbol has the same significance as the "negative free water clearance" of Wesson and Anslow (9), and the "distal water economy" of Brodsky, Rapoport, and West (10). The symbol $Tm^{C}_{H_2O}$ (Figure 2) is meant to represent the constant maximum level of solute free water removed during the concentrating process at wide ranges of urine flow above a minimal level (e.g., dog No. 415, Control periods, Table IV).

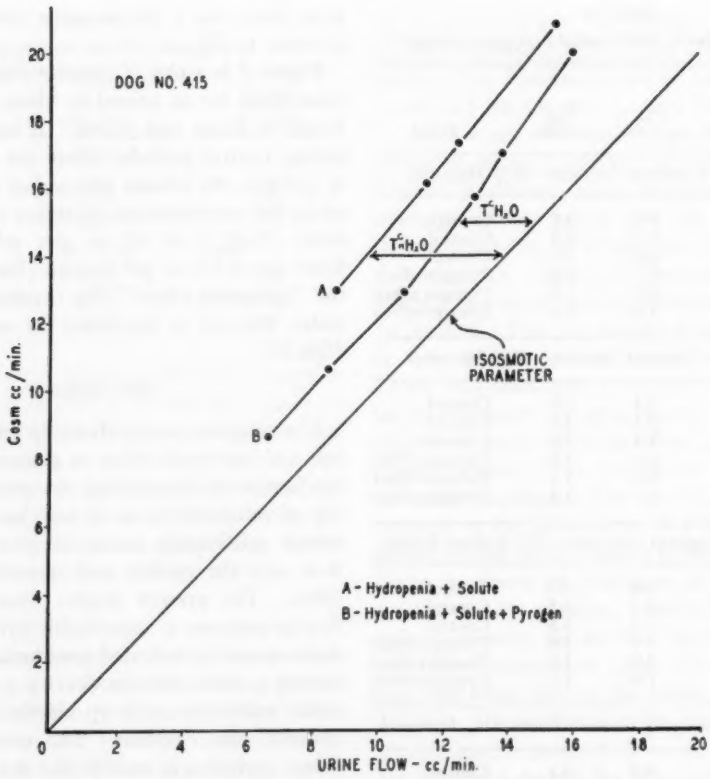

FIG. 2. A PLOT OF URINE FLOW AGAINST C_{osm} IN A HYDROPENIC SOLUTE LOADED DOG BEFORE AND AFTER PYROGEN

(increased filtered load) and a more prolonged tubular factor to account for the electrolyte excretion pattern during the pyrogenic effect. In dogs receiving a glucose and water infusion (Group III), electrolyte diuresis is less marked and transitory and follows the time course of changes in filtration rate.

Recent work has indicated that one of the effects of the administration of pyrogen is a diminution in adrenal hormone production (12). An altered adrenal "salt" steroid production induced during the pyrogenic reaction may not explain the increased urine electrolyte excretions completely, since a rise in excretion of solute, mainly electrolyte, is seen in adrenalectomized dogs given pyrogen (13). The possibility that pyrogens may produce their effects on electrolyte excretion through alterations in the paraventricular nuclei with a resulting natriuresis is not known at this time (14).

The uniform rise in total excreted solute following the administration of pyrogen does not in itself account for the uniform rise in minute flow of urine. It is apparent from Group I and Group III dogs that a dissociation between the renal handling of solute free water and solute water exists and that they may vary independently in response to pyrogens. The fall in osmotic U/P ratio, the rise in free water clearance (negative towards positive) and the rise in osmolar clearance in normally hydrated dogs of Group I is supporting evidence. Furthermore, in animals of Group III during the inhibition of Pitressin® by pyrogen urine flow increased without an increase in solute excretion. It is apparent from the Group III dogs that effective doses of pyrogen are capable of blocking fully antidiuretic doses of Pitressin®. Once established, even excessive doses of Pitressin® will not alter the augmented urine flow of a py-

rogen diuresis in water loaded animals. Thus, the effect of exogenous Pitressin® can be blocked by an effective dose of pyrogen.

Recent studies by del Greco and de Wardener (15), and Berliner and Davidson (16) have shown that under certain circumstances, in the absence of endogenous ADH of water loading, dogs may elaborate a concentrated urine; further, these studies have implicated a fall in filtration rate and the rate at which solute is delivered to the concentrating apparatus, as possible factors. Recalculation of published data from solute loaded-hydropenic dogs in which "diabetes insipidus" was produced acutely by surgical means shows that such animals elaborate an isotonic urine at all rates of solute excretion (17); but under prolonged observation dogs made "diabetes insipidus" by surgical means may elaborate concentrated urine during hydropenia (18). Thus, at the two extremes of hydration the evidence indicates that posterior pituitary antidiuretic hormone is not the sole factor controlling the concentration of urine.

The biological similarity of exogenous ADH (Pitressin®) and endogenous posterior pituitary ADH in their ability to produce a concentrated urine under conditions of solute loading has been demonstrated (19). Two mechanisms may explain the interference with the maximum reabsorption of solute free water in hydropenic-solute loaded dogs by pyrogen (see Figure 2, line B): 1. It can be assumed from the observations in Group III dogs that the concentrating effect of endogenous ADH has been eliminated by pyrogen in Group IV dogs. The horizontal distance between line B and the isosmotic parameter, Figure 2, represents antidiuretic factors other than ADH which are not effected by pyrogen. 2. A decreased filtration rate and solute delivery to the concentrating segment is associated with increased free water reabsorption (15, 16). The corollary may follow that the increased filtration rate (and solute delivery) usually seen during the pyrogen effect evokes an opposite effect of decreased reabsorption of free water. Our evidence supports the first explanation since the requirement of increased solute excretion necessary for the second explanation was not found in our dogs (Group IV) at the time of the fall in free water reabsorption ("pyrogenic effect").

Evidence of increased salt excretion in humans during the infusion of pyrogenic inulin and triple typhoid (preceded by a transient fall with typhoid) is known (20, 21). The undesirable side-effects of pyrogens (e.g., fever, abdominal cramps, etc.) in their present form limit their clinical usefulness when given in comparable doses to those described for the dog. Whether varying degrees of the phenomena reported here are common to all fever-producing agents and especially the endotoxins of gram negative bacteria is not known. Fever was not uniformly seen in the present studies; when present, its prevention by antipyretics, as in other studies with pyrogen, did not alter its renal effect (22). While no substance with the effects reported here has been described, the possibility that a polysaccharide (from gram negative bacteria) which might be clinically useful needs further investigation.

SUMMARY AND CONCLUSIONS

1. An increase in solute excretion and interference with the concentrating ability of the dogs' kidney has been demonstrated to be the result of the administration of a large dose of pyrogen.

2. Observations on the renal effects of pyrogen were made in 21 dogs, divided into four groupings. The following effects were noted: A. In dogs receiving a slow saline infusion, there was a uniform and often significant rise in urine flow, renal plasma flow, osmolar clearance and excretion of solute, mainly sodium, potassium and chloride. Inconsistent changes in filtration rate were noted as a result of pyrogen administration. There was also a uniform fall in osmotic U/P ratio and a decrease in "negative free water" clearance. B. In six dogs tubular maxima for glucose and PAH were studied before and after pyrogen administration and found to give inconsistent and variable results. C. The administration of pyrogen to dogs in whom an initial water diuresis had been inhibited by Pitressin® showed a resumption of the diuresis in spite of continued Pitressin® administration. This resumption of diuresis was not accompanied by an increased osmolar clearance, but rather by a dilute urine having a "positive free water" clearance. D. In hydropenic, solute-loaded dogs, $Tm^{C}_{H_2O}$ was reduced by about half.

3. The results, their interpretation and possible clinical application were discussed.

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CERTAIN ASPECTS OF TYROSINE METABOLISM IN THE YOUNG. II. THE TYROSINE OXIDIZING SYSTEM OF FETAL RAT LIVER [1, 2]

By NORMAN KRETCHMER and HELEN McNAMARA

(From the Department of Pediatrics, The New York Hospital-Cornell Medical Center, New York, N. Y.)

(Submitted for publication March 15, 1956; accepted May 11, 1956)

A recent study and review (1) of tyrosine metabolism has shown that the overall oxidation of tyrosine to acetoacetate and fumarate requires two moles of oxygen. The first step is the conversion of tyrosine to p-hydroxyphenylpyruvate by a non-oxidative transamination. The p-hydroxyphenylpyruvate is in turn oxidized to homogentisate with the utilization of one mole of oxygen. This reaction requires the presence of a reducing agent such as ascorbic acid (2–5) or dichlorophenolindophenol (6, 7). Recent data (8) indicate that the conversion takes place without the previously postulated (9) formation of an intermediate such as 2,5-dihydroxyphenylpyruvate. The homogentisate is oxidized by one mole of oxygen to maleylacetoacetate in the presence of reduced iron, and the maleylacetoacetate is further acted upon by an isomerase to yield fumarylacetoacetate. Finally, enzymatic hydrolysis of this compound results in the formation of the end products, fumarate and acetoacetate.

This metabolic pathway is of special interest to pediatricians (10–12) since it has been shown (10) that p-hydroxyphenylpyruvic acid and tyrosine are excreted in the urine of premature infants maintained on diets high in proteins but not supplemented with vitamin C. It has also been shown (11) *in vitro,* that there is considerably less tyrosine oxidizing activity in the liver of the premature infant than in the liver of the human adult or full-term infant. This deficiency was ascribed to a decrease in the concentration of the transaminase apoenzyme.

The present investigation indicates a similar deficiency in the liver of the fetal rat. Paucity of tyrosine oxidation in the fetal liver of this animal can also be explained by a lack of specific tyrosine transaminase apoenzyme. The possible relationship of these findings to the so-called "inborn errors of metabolism" will be discussed.

METHODS

Rats of the Long-Evans strain were used throughout the study. Adult rats were killed by a blow to the head and the liver was immediately removed and chilled. Infant rats were killed by decapitation to facilitate exsanguination. Newborn rats were obtained as they were born. Fetal rats were obtained as near to predicted term as possible. The mother rat was killed by a blow to the head and her gravid uterus was excised and immediately placed on ice in the cold room. The fetuses were separated from the placentae and membranes, decapitated and the livers removed.

Litter mates that were allowed to survive were divided into two groups each comprising two or three rats. One group was nursed by the mother. The other group was taken from the mother, fed only water by tube and kept in cotton batting for warmth. At necropsy all of the nursing animals had milk in their gastro-intestinal tracts.

All procedures were carried out in a cold room (temperature 2° to 4° C). The livers from two to four fetal or infant rats were usually pooled in order to obtain a sample adequate for analysis.

The pooled livers from fetal and infant rats were ground for two minutes in a chilled Teflon-glass homogenizer with the dropwise addition of one to three volumes of ice-cold 0.9 per cent KCl. The livers from adult rats were ground for two minutes in a Waring Blendor with three volumes of the cold salt solution.[3] All of the homogenates were spun at 18,000 × g for 15 minutes in the Sorvall SS-1 centrifuge. The supernatant fluid derived from the high-speed centrifugation (soluble fraction) was

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[2] Presented in part before the American Physiological Society, Atlantic City, April 18, 1956.

[3] All the livers were homogenized and analyzed immediately after removal. It was found that quick freezing and storing at − 40° C before analysis resulted in a marked depression in tyrosine oxidizing activity. For example, newborn rat livers analyzed immediately gave a value of 35 μl. O_2 per 30′ per mg. N. whereas frozen and stored newborn rat livers gave a value of 8 μl. O_2 per 30′ per mg. N.

assayed for tyrosine transaminase and overall tyrosine oxidizing activities.

The manometric procedure as outlined by Knox and LeMay-Knox (2) was followed in the assay of overall tyrosine oxidation. The flask contents were essentially those previously described (11): 2.0 ml. of 0.2 M phosphate buffer pH 7.4 with or without 8 μM. of tyrosine, 0.2 ml. of a solution containing 500 μg. of ascorbic acid neutralized to pH 7.4. The sidearm contained 0.3 ml. of α-ketoglutaric acid (30 μM.) adjusted to pH 7.4 and 0.2 ml. of pyridoxal phosphate in water (60 μg.). The center well had 0.2 ml. of 20 per cent KOH on a strip of filter paper. The total volume was adjusted to 3.7 ml. with glass-distilled water. For assay of tyrosine transaminase the flask contents were the same except that ascorbic acid was omitted and flasks were incubated in an atmosphere of nitrogen to prevent oxidation of the formed p-hydroxyphenylpyruvate.

In all instances, the flasks were equilibrated for a period of ten minutes at 37.3° C. in an atmosphere of either air or nitrogen. Stopcocks were then closed and the contents of the sidearms added. Readings were taken at intervals of ten minutes for a period of sixty minutes. Control flasks containing equivalent amounts of tissue, but no tyrosine, were always run along with the experimental flasks and endogenous oxidative activity was subtracted from values obtained with the experimental flasks.

Nitrogen was determined on the original liver preparation by the Ma-Zuazaga modification of the micro-Kjeldahl method (13). At the end of the incubation period in air separate aliquots were taken from the flasks and added either to trichloroacetic acid or metaphosphoric acid to yield a final concentration of 5 per cent acid. The samples incubated in nitrogen were added only to trichloroacetic acid. The supernatant fluid resulting from the precipitation with trichloroacetic acid was used for the determination of Brigg's reacting material (p-hydroxyphenylpyruvic acid when the samples were incubated in nitrogen). The supernatant fluid from the metaphosphoric acid precipitations was used for the determination of total p-hydroxyphenyl compounds as previously reported (11) in order to check oxygen uptake.

RESULTS

The data presented in Table I show the activity of the tyrosine oxidizing system as obtained with the soluble fraction of the livers of rats of different ages. The activity of fetal liver was about 12 times less than that of newborn fullterm liver on the basis of either wet weight or nitrogen. The age difference between newborn and fetal rats was no more than one to three days. The liver of the newborn rat averaged 70 per cent of the activity of adult liver on the basis of nitrogen; and 50 per cent on the basis of wet weight. The activities of the livers of the pregnant and adult rat were the same.

TABLE I

Tyrosine oxidizing activity of the soluble fraction of rat liver

	No.	Range μl./30'/mg. N	Ave.	Range μl./30'/gm. wet wt.	Ave.
Fetal rats	3*	2.0–3.6	2.7	32–40	35
Newborn rats	5*	19–67	35	252–812	538
Adult rats	6	29–73	52	760–1,472	1,183
Pregnant rats	2	29–60	45	944–1,424†	1,225

* This refers to the number of separate pools of livers rather than individual livers.

† This range represents the oxidative activity obtained from the livers of three individual rats.

The effect of postnatal feeding on the activity of the tyrosine oxidizing system was studied. Infant rats were killed at different intervals following birth and activities were determined in the soluble fraction of the livers.

The data in Figure 1 show that the tyrosine oxidizing activity of fetal liver was minimum (3 μl. O_2 per 30' per mg. N.) in comparison to any other age group. The tyrosine oxidizing activity of the livers of newborn rats which had nursed was 43 per cent less than the activity of the livers of rats immediately at birth. The activity in non-nursed litter mates of similar age approximated that of newborn rat liver and was nearly twice as high as that of their nursing litter mates. By 24 to 48 hours of age the activity of the infant rat liver was

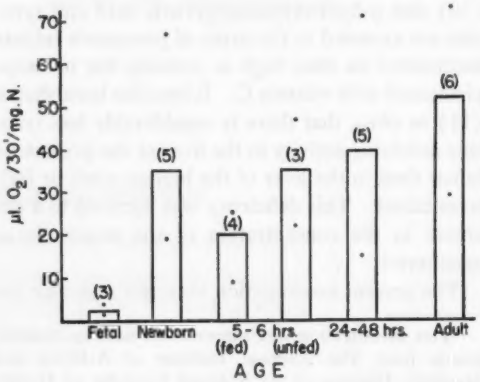

FIG. 1. THE TYROSINE OXIDIZING ACTIVITY OF RAT LIVER IN RELATION TO AGE

The black dots indicate extremes. The numbers in parentheses represent the number of pools of livers. In the case of the adult only individual livers were used.

above the newborn level and averaged 83 per cent of the activity of the adult rat.

Detailed observations on the influence of nursing are shown in Figure 2 where groups of rats from the same litter are compared. The data indicate that the overall tyrosine oxidizing activity in the liver of non-nursed rats reached considerably higher levels than those of the liver of nursed animals.

The activity-time curves presented in Figure 2 would indicate that a value taken at any particular time is less meaningful than an examination of the entire time course of oxygen uptake.

At the end of 30 minutes of incubation the liver of the non-nursed rats took up 28 to 100 per cent more oxygen than did the liver of the nursed rats. After 60 minutes of incubation there was 65 to 143 per cent more oxygen uptake with the liver of the non-nursed rats. However, there was progressive increase in oxygen uptake by the liver of newborn rats with time whereas the oxygen uptake of the liver of rats 5 hours old reached a sustained plateau in 20 minutes or even fell off thereafter.

Since the first and obligatory step in tyrosine oxidation is its non-oxidative transamination to p-hydroxyphenylpyruvic acid, it was postulated in a previous paper (11) that the lesser overall tyrosine oxidizing activity of premature infant and perhaps fetal rat liver might in part be due to deficiency in tyrosine transaminase apoenzyme.

Figure 3 represents the amount of Brigg's reacting material (p-hydroxyphenylpyruvic acid) produced by the soluble fractions of fetal and adult

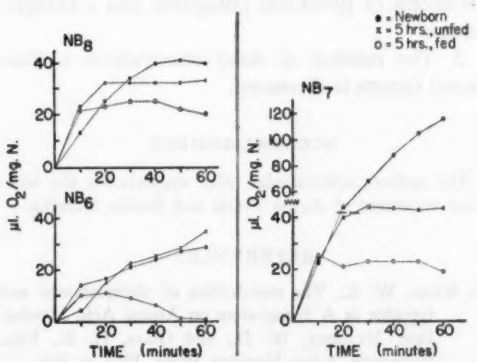

FIG. 2. TYROSINE OXIDIZING ACTIVITY IN THE LIVER OF THE NEWBORN RAT: EFFECT OF FASTING

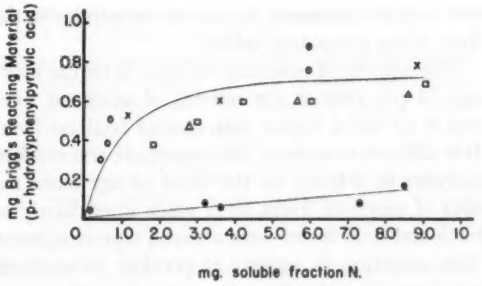

FIG. 3. ACTIVITY OF TYROSINE TRANSAMINASE IN FETAL AND ADULT RAT LIVER

Each different symbol represents separate determinations using the same rat liver or a pool of livers. Adults are represented as ○, ×, △, □; fetuses are represented as ●, ⊗. These lines are drawn for better visualization.

rat livers. Increasing quantities of soluble fraction were incubated with tyrosine for 60 minutes in an atmosphere of nitrogen. In each instance the necessary cofactors, α-ketoglutarate and pyridoxal phosphate, were added in excess. Under these conditions the amount of soluble fraction determines the amount of apoenzyme and the results indicate that the tyrosine transaminase in fetal liver is of the order of 12 times less than that of adult rat liver when 3.0 to 4.0 mg. of soluble fraction nitrogen are used. The remaining enzymes comprising the tyrosine oxidizing system are now being tested in similar fashion.

DISCUSSION

The data herein presented demonstrate that the activity of the tyrosine oxidizing system is markedly low in fetal rat liver. This minimum activity, measured in the presence of excess cofactors, must be ascribed to a diminished amount or activity of tyrosine transaminase apoenzyme. Under physiologic conditions it is possible that the activity of the transaminase would be even lower than these data indicate since there may be an additional deficiency of the necessary cofactors including pyridoxal phosphate.

Since transamination is the first obligatory reaction, a low concentration or activity of tyrosine transaminase would of necessity result in a low overall tyrosine oxidizing activity. However, data previously presented (11) suggest that all apoenzymes involved in this pathway of tyrosine oxida-

tion may be decreased in amount or activity in the liver of the premature infant.

The activity of newborn rat liver is on the average 74 per cent of the activity of adult rat liver and is 12 times higher than that of fetal rat liver. It is difficult to explain this immediate and marked increase in activity on the basis of age since assays of newborn livers were made immediately at birth and fetal livers were assayed almost at term. This elevation in activity is peculiar to newborn rat liver since the liver from nursing infant rats 5 to 24 hours old showed an activity about one-half that of newborn rat liver. However, deprivation of food from rats of this age resulted in an increase in tyrosine oxidizing activity as measured after 30 minutes of incubation. One possible explanation for these results is that the stress of the birth process led to the release or activation of a hormone or other substance influencing tyrosine oxidation. The persistence of high activity in the livers of infant rats who were not nursed could then be explained by the continuing hormonal stimulation resulting from the additional stress of fasting. This hypothesis receives support from the fact that the induced tyrosyluria of premature infants can be alleviated by the administration of ACTH (14). It is being tested by the administration of pituitary, adrenal cortical and medullary hormones [4] to pregnant rats.

The manifestation or appearance of a particular enzyme activity, at a rate which would be too rapid to result from *de novo* synthesis, could be explained by the activation or unmasking of an inactive form of an enzyme. The special cases of the proenzymes or zymogens of the digestive enzymes are examples of this phenomenon. In addition, other varieties of enzymes exhibiting a similar type of behavior have recently been described, such as the rapid *in vivo* activation of phosphorylase during the course of muscle stimulation (15), the *in vitro* activation of nucleotidases and phosphatases by temperature (16), the hormonal activation of muscle and liver phosphorylase (17) and tryptophan peroxidase (18).

Certain enzyme activities have been reported to be diminished in the organs of the fetus (19–21). Usually, deficiencies in enzyme activity in later life are associated with distinct disease entities. For example, in the liver of patients with glycogen storage disease there is commonly a diminished glucose-6-phosphatase activity (22). Due to this low activity, glycogen can be formed but cannot be converted to glucose. It has also been reported (23) that the liver of a premature infant had a similar low level of glucose-6-phosphatase activity. Nemeth (19) also presented evidence that the glucose-6-phosphatase activity of the livers of guinea pigs increases with the age of the animals. Thus, some of the more clearly identified disorders related to specific enzyme deficiencies or inactivities may stem from a persistence of fetal or embryonic enzymic states resulting from genetic (24) or congenital biochemical influences.

[4] Preliminary experiments in this laboratory have demonstrated that there is no *in vitro* stimulation of tyrosine oxidation by the addition of cortisone, hydrocortisone or ACTH to the liver homogenate or to the soluble fraction of liver.

SUMMARY

1. The tyrosine oxidizing activity of fetal rat liver is 12 to 15 times lower than that of fullterm or adult rat liver.

2. At birth the tyrosine oxidizing activity of the newborn liver approaches that of adult rat liver.

3. When postnatal rats are unfed, their livers have a tyrosine oxidizing activity approximately equal to that found in the liver of the newborn rat, in contrast to the livers of fed infant rats which show an activity about one-half that of the liver of newborn and unfed rats.

4. The lack of overall tyrosine oxidation by fetal rat liver can be explained by a lack of tyrosine transaminase activity, even in the presence of an excess of pyridoxal phosphate and α-ketoglutarate.

5. The relation of these observations to hormonal factors is discussed.

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CHANGES IN BODY COMPOSITION IN ACUTE RENAL FAILURE[1]

By L. W. BLUEMLE, JR.,[2] H. P. POTTER, AND J. R. ELKINTON[3]

(From the Chemical Section of the Department of Medicine, University of Pennsylvania, School of Medicine, Philadelphia, Penna.)

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To a large degree the therapy of acute renal failure depends on an understanding of biochemical disturbances that result from the temporary absence of renal excretory and homeostatic functions. Important among these disturbances are changes in the volume and composition of body fluids, particularly with respect to electrolyte constituents. While certain manifestations of these changes are frequently recognized on the clinical level, relatively little quantitative data are available from which can be derived a general pattern of body fluid alterations in this disease. One reason for this paucity of information, aside from the limitations of investigative techniques, is the inherent difficulty of performing adequate studies on critically ill patients.

Sirota and Kroop (1) observed in 4 oliguric patients an expansion of inulin space roughly commensurate with estimated positive fluid balance. They postulated that the oliguric phase of acute renal failure is associated with cellular dehydration resulting from a shift of water out of cells, and that hyponatremia may result in part from extracellular dilution and in part from a shift of sodium into cells. The observation by Schwartz, Tomsovic, and Schwartz (2) of greater expansion of inulin space than of D_2O space in one anuric child would conform with this concept. Iseri, Batchelor, Boyle, and Myers (3) also suggested that the hyponatremia and hypochloremia of oliguria may be due in part to cellular uptake of sodium and shift of water from cells to extracellular fluid.

The role of catabolism in augmenting total body water through water of oxidation and release of preformed water was evaluated by Hamburger and Richet (4), largely by inference from observations made during post-oliguric diuresis. Changes in the volume and distribution of body water were also studied in anuric dogs by Hamburger and Mathé (5), who found close agreement between the expansion of total body water as measured by D_2O spaces and the amount of water derived from metabolic processes. On the basis of extensive clinical observation and a limited number of isotopic dilution studies, Merrill (6) has enumerated some of the fluid and electrolyte changes frequently seen in prolonged acute renal failure, as follows: 1) an increase in total body water, 2) an increase in total body sodium with a decreasing serum concentration, 3) a decrease in total body potassium with an elevated serum level, and 4) an increased extracellular fluid volume.

The primary purpose of the present study was to obtain more complete information concerning the type and magnitude of changes in volume and electrolyte composition of body fluids in acute renal failure, to assess the roles of catabolic processes and concomitant fluid therapy in their genesis, and to delineate certain therapeutic implications of this information.

EXPERIMENTAL MATERIAL AND METHODS

Eight patients with acute renal failure were studied by the balance technique on the Metabolic Unit of the Hospital of the University of Pennsylvania. Studies were initiated in 13 patients but were interrupted or invalidated in 5 for various reasons. Measurements were made of intake of water, solids, chloride, sodium, potassium, nitrogen, and carbohydrate and fat, and of all output of water, solids, chloride, sodium, potassium, and nitrogen. At the beginning and end of each unit balance period (24 to 72 hours) body weight was obtained on a stretcher-scale (7) and blood was drawn for determinations of serum chloride, sodium, potassium, CO_2 content, and blood urea nitrogen. Chemical methods of these analyses have been previously described (8). On

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[2] J. Allison Scott Fellow of the Department of Research Medicine (1953–55); Markle Scholar in Medical Science 1955–56.

[3] Established Investigator of The American Heart Association.

TABLE I

Summary of clinical material

Patient	Age	Sex	Body surface area* (*sq. meters*)	Apparent cause of acute renal failure	Complicating factors	Duration of oliguria (*days*)	Duration of study (*days*)	Day of dialysis (from onset of oliguria)	Result
V. N.	42	F	1.82	Hemolytic reaction to 1000 ml. incompatible blood during hysterectomy	Acute parotid abscess during diuretic phase	12	24	7	Recovered
E. G.	41	F	1.59	Hemolytic reaction to 2000 ml. incompatible blood during hysterectomy	Pelvic peritonitis, convulsions during diuretic phase	12	22	7	Recovered
B. S.	26	F	1.55	Septic abortion	Peritonitis, pelvic abscess, GI hemorrhage, chemical pericarditis, pul. congestion	12	15	10	Recovered
K. B.	42	F	1.73	Hemolytic reaction to 2000 ml. incompatible blood during cholecystectomy	Convulsions during diuretic phase	14	19	8	Recovered
I. T.	60	F	1.72	Hypotension during facial surgery for carcinoma	Hemorrhage from operative site during diuretic phase	17	22	8	Recovered
M. G.	43	F	1.89	Hypotension following hysterectomy	Pulmonary edema, potassium intoxication	9	3	None	Died
F. B.	58	F	1.47	Hypotension during cardiac surgery	Shock following dialysis	10	12	9	Recovered
D. S.	24	F	1.74	Postpartum shock and hemolytic reaction to 500 ml. incompatible blood	Convulsions during diuretic phase	10	14	9	Recovered

* Surface area in sq. meters = $Wt.^{0.425} \times Ht.^{0.725} \times 0.007184$ (after DuBois and DuBois [15]).

completion of each study the observed data were analyzed for each unit balance period and recorded for the sake of convenience as average values per 24 hours for each of the following three phases: I) the oliguric phase (urine volume less than 400 ml. per 24 hours), II) the early diuretic phase (urine volume 400 ml. per 24 hours to maximal diuresis), and III) late diuretic phase (maximal diuresis to end of study). In all except one case (M. G.) balance studies were continued until the patient was ambulatory and was receiving no special therapy. It should be clearly pointed out that balance studies were not inititated at the onset of oliguria in any of these patients, the average interval being 8.4 days, the shortest 5 days, between onset of oliguria and beginning of studies. Hence the cumulative data relating to Phase I should *not* be considered representative of metabolic changes during the *entire* oliguric period.

Brief clinical summaries of each case are presented in Table I. All patients were transferred from other hospitals in either the latter part of the oliguric phase or the early diuretic phase. Duration of study ranged from 3 to 24 days (average 16.4 days). Four patients were studied during Phases I, II, and III; two during only Phases II and III; one during only Phases I and II, and one during only Phase I. All patients were critically ill at the time of transfer, and all except one (M. G.) were treated by extracorporeal hemodialysis prior to initiation of balance studies.[4] No attempt was made to determine the effect of dialysis on subsequent fluid and electrolyte balance; however, the predominant effect as judged by improvement in serum electrolyte pattern and, in some cases, loss of excess body water (by ultrafiltration) was to compensate partially for the fluid and electrolyte disturbances which had occurred up to that time.

Therapy prior to transfer to our hospital varied considerably from patient to patient. In almost all cases little attempt had been made to restrict fluid intake during the early days of oliguria; in 3 patients (B. S., I. T., and M. G.) signs of overhydration were evident on admission. Fluid therapy during this study, presented as average intake figures in Table II, followed generally accepted principles of attempting to prevent or correct the more

[4] In one case (K. B.) dialysis was performed on the third day of study, and this day was omitted from the calculations.

serious fluid and electrolyte disturbances as judged by clinical observations and serum studies. Balance data were not used as the primary criteria for judging subsequent fluid therapy. During the oliguric phase intake was limited to an average of 1,000 ml. per day, or roughly 700 ml. plus previous 24-hour measured water loss, except in one case (M. G.) where pulmonary edema necessitated more drastic fluid restriction. Electrolyte intake was also limited during this phase because of minimal output. Both water and electrolyte intake were increased during Phases II and III to compensate for increasing urinary losses.

CALCULATIONS

Changes in the composition of the body and in certain constituents of the body fluids may be calculated from the data obtained by the balance technique as described above. Although most of these derivations have been in use for many years, the equations which were used in this study are presented here in order that our analysis may be clearly understood. The validity of the data so derived is taken up subsequently under *Discussion*.

Metabolic mixture

Carbohydrate burned (C) was assumed to be equivalent to the carbohydrate administered.

Protein burned (P) in grams was calculated from the nitrogen excretion in the urine (UV_N) in grams corrected for changes in the amount of the urea nitrogen in the body fluids (ΔBUN) in grams:

$$P = 6.25 \times (UV_N + \Delta BUN). \quad (1)$$

This latter correction, a proportionately large one in the severely oliguric patient, was made by multiplying the change in concentration of blood urea nitrogen ($\Delta[BUN]$), in grams per liter, by an assumed volume for total body water (W) in liters:

$$\Delta BUN = \Delta[BUN] \times W. \quad (2)$$

Since change in the last factor, W, is one objective of the calculation, a series of approximations beginning with a total body water assumed to be 73.2 per cent of the lean body mass at the end of the study, permits a reasonable calculation of this portion of the protein burned.

Fat burned (F) was calculated from the insensible weight loss (IL) and the carbohydrate and protein burned, according to the formula of Lavietes (9):

$$F = (IL - 2.12\ C - 1.69\ P)/3.79, \quad (3)$$

where

$$IL = (\text{Wt. intake} - \text{Wt. output}) - \Delta Wt. \quad (4)$$

Change in body composition

Change in body fat (ΔBF) was taken as the difference between the exogenous fat given (F_{ex}) and the fat burned (F):

$$\Delta BF = F_{ex} - F. \quad (5)$$

Change in total body water (ΔW) was calculated from the change in weight (ΔWt) corrected for the balance of solids ($S_{excreta} - S_{ingesta}$) and the metabolic mixture (10):

$$\Delta W = \Delta Wt + (S_e - S_i) + (C + F + 0.54\ P). \quad (6)$$

Change in total body solids (ΔBS) was taken as:

$$\Delta BS = \Delta Wt - (\Delta BF + \Delta W). \quad (7)$$

Change in lean body mass (ΔLBM) was obtained as:

$$\Delta LBM = \Delta Wt - \Delta BF \text{ or} \quad (8)$$

$$= \Delta W + \Delta BS. \quad (9)$$

In addition, absolute amounts of the principal body components were estimated in each patient at the end of the study when the body constituents presumably had returned to or toward normal proportions. Lean body mass (LBM) was calculated in all but one case (M. G.) from the excretion rate of creatinine (UV_{cr}) during the last 2 or 3 days (when the plasma creatinine had fallen to essentially normal levels), by the formula of Miller and Blyth (11):

$$LBM = 20.97 + 0.5161\ UV_{cr}, \quad (10)$$

where UV_{cr} is the excretion rate of creatinine in milligrams per hour, corrected for any change in plasma level (in the same manner as UV_N is corrected in Equation *2* above). Given the estimated lean body mass, total body fat (BF), total body water (W), and total body solids (BS) are calculated as:

$$BF = WT - LBM, \quad (11)$$

$$W = 0.732\ LBM, \quad (12)$$

$$BS = LBM - W. \quad (13)$$

Given these absolute values, changes in total body fat, water, and solids were readily calculated backward in time from the end of the study by using the values for changes derived in Equations *5*, *6*, and *7* above.

Change in extracellular fluid volume (ΔE_{Cl}), as equated with the chloride space, was calculated from the chloride balance in the usual manner (12) and *change in intracellular fluid* (ΔI) was taken as the difference between ΔW and ΔE_{Cl}. For purposes of calculation of percentage changes the total extracellular water at the end of the study was assumed to be 22.5 per cent of the lean body mass.

Electrolyte exchanges

Changes in extracellular sodium and potassium (ΔNa_E and ΔK_E) and in intracellular sodium and potassium (ΔNa_I and ΔK_I) were calculated on the basis of the chloride space in the usual manner as described in Equations *20* to *23* inclusive in Elkinton and Danowski (13). Transfers of intracellular potassium in excess of nitrogen ($\Delta K_I'$) were calculated on the assumption of a normal K:N ratio of 3.0 mEq. to 1 gm. Electrolyte losses through sweat were not included in the calculations, this loss being considered insignificant since the patients were kept in air-conditioned rooms.

TABLE II

Observed data: Intake and output of water, total solids, electrolytes and nitrogen

Patient	Study periods (days from onset of oliguria)	Phase†	Average intake per 24 hours								Average output per 24 hours											
											Urine							Stools, *etc.**					
			H_2O	Solids	Cl	Na	K	N	CHO	Fat	Vol.	Solids	Cl	Na	K	N	Creat.‡	H_2O	Solids	Cl	Na	K	N
			gm.	*gm.*	*mEq.*	*mEq.*	*mEq.*	*gm.*	*gm.*	*gm.*	*ml.*	*gm.*	*mEq.*	*mEq.*	*mEq.*	*gm.*	*mg.*	*gm.*	*gm.*	*mEq.*	*mEq.*	*mEq.*	*gm.*
V. N.	9–12	I	1,278	143	0	1	1	0.1	126	20	273	7	17	20	7	1.1		94	6	11	7	0	0.8
	13–20	II	2,349	230	141	156	24	0	216	0	2,490	56	165	197	50	10.4		82	21	8	5	0	0.5
	21–32	III	3,006	283	146	146	74	4.5	226	28	2,604	52	151	152	72	13.4	765	228	28	7	8	16	1.5
E. G.	9–12	I	1,424	101	0	20	0	0	99	0	230	5	15	21	3	0.8		145	10	9	8	4	0.8
	13–21	II	2,462	107	64	77	25	0.1	97	1	1,918	34	68	106	29	6.1		153	8	9	13	4	1.2
	22–30	III	3,356	220	112	126	56	1.4	153	10	2,292	46	72	112	67	7.3	918	151	14	6	7	9	0.8
B. S.	12	I	1,144	105	0	0	0	0	105	0	230	7	13	19	6	1.4		115	19	3	6	16	1.4
	13–19	II	2,832	240	68	88	23	1.0	186	0	2,231	48	101	157	46	9.8		224	24	30	9	5	0.9
	20–26	III	3,398	226	154	165	78	1.3	187	11	2,495	71	86	156	72	14.6	881	314	50	34	23	14	3.6
K. B.	6–14	I	915	132	0	0	1	0.1	86	4	94	4	9	11	4	0.7		194	29	7	14	19	0.7
	15–21	II	2,381	209	38	74	14	0.1	192	2	1,919	34	66	92	49	11.4		153	23	7	8	8	0.8
	22–24	III	3,152	268	80	73	59	1.7	212	12	2,493	47	66	95	65	16.3	749	13	2	2	3	0	0
I. T.	10–17	I	1,129	185	3	5	7	0.1	141	30	190	5	11	17	6	0.8		59	12	2	2	1	0.2
	18–31	II	2,692	314	79	84	39	0.2	218	66	1,580	30	61	82	39	5.1	539	56	16	1	3	6	0.6
M. G.	7–9	I	285	134	0	13	0	0	132	0	92	2	6	8	4	0.6	—	19	1	1	1	0	0.2
F. B.	11–18	II	2,865	174	137	124	93	3.1	137	24	2,446	51	129	140	91	8.7		67	19	4	7	5	1.1
	19–22	III	2,934	190	104	97	103	5.2	130	32	2,294	60	116	118	106	11.0	412	55	21	1	2	8	0.8
D. S.	11–19	II	3,517	327	119	146	31	4.1	229	56	3,519	47	127	215	52	11.9		89	12	10	10	6	0.6
	20–24	III	4,850	371	130	140	84	9.7	240	86	4,065	47	116	147	64	14.7	772	168	34	2	19	14	1.0
Total averages	8–13	I	1,028	121	1	7	2	0.1	115	9	185	5	12	16	5	0.9		104	13	6	7	7	0.7
	14–22	II	2,728	228	92	107	36	1.4	182	21	2,300	43	102	141	51	8.3		118	18	10	8	5	1.6
	23–29	III	3,463	260	121	125	76	4.0	191	29	2,707	54	101	130	74	12.9	719	155	25	9	11	10	1.3

* Includes blood drawn for analysis and vomitus and drainage when present.
† See text for definition.
‡ At end of study, corrected for change in serum creatinine concentration.

TABLE III

Observed data: body weights, total balances of electrolytes and nitrogen, concentrations of electrolytes in serum and of blood urea nitrogen

Patient	Day from onset of oliguria	Total balance*				Body weight	Serum concentration				Blood urea nitrogen
		Cl	Na	K	N		Cl	Na	K	CO_2	
		mEq.	*mEq.*	*mEq.*	*gm.*	*kg.*	*mEq. per L.*	*mEq. per L.*	*mEq. per L.*	*mM. per L.*	*mg. per 100 ml.*
V. N.	8					79.43	97	139	5.1	28.7	81
	12	−109	−106	− 22	− 7.5	79.08	92	133	5.2	21.8	118
	20	−256	−374	−213	− 87.4	72.48	91	138	4.0	25.0	148
	32	−133	−177	−156	−124.8	68.50	100	139	4.0	31.0	11
E. G.	8					61.96	88	133	3.9	30.2	66
	12	− 92	− 32	− 25	− 6.3	62.21	77	128	3.9	27.6	114
	21	−119	−386	− 73	− 64.4	55.04	87	136	4.9	25.5	82
	30	+309	+ 66	−178	− 59.9	53.76	103	141	4.8	21.7	15
B. S.	11					59.50					122
	12	− 16	− 25	− 22	− 2.8	59.47	88	138	4.8	24.6	142
	19	−440	−550	−203	− 68.2	55.04	76	131	3.5	25.4	180
	26	+232	−100	− 56	−118.9	51.66	105	140	4.0	25.8	33
K. B.	5					63.70	93	134	4.7	17.5	120
	14	−124	−196	−170	− 10.4	60.01	91	136	5.4	14.9	175
	21	−247	−180	−298	− 84.4	57.14	91	136	3.7	30.0	116
	24	+ 39	− 72	− 18	− 43.9	57.03	90	135	3.8	29.3	70
I. T.	9					68.42	100	146	5.6	28.3	36
	17	− 81	−118	+ 3	− 7.5	66.84	94	141	6.3	20.5	140
	31	+238	− 16	− 90	− 77.4	62.97	99	137	4.3	26.4	34
M. G.	6					79.90	87	134	6.8	19.5	132
	9	− 21	+ 15	− 11	− 2.4	77.48	91	139	10.8	10.8	248
F. B.	10					45.10	85	131	4.2	25.5	80
	18	+ 32	−183	− 29	− 53.3	39.60	104	143	4.0	25.8	72
	22	− 53	− 89	− 40	− 26.6	39.06	102	138	4.0	25.6	39
D. S.	10					67.13	84	134	6.3	26.5	112
	19	−162	−708	−253	− 75.2	59.32	103	141	3.7	18.9	72
	24	+ 63	−135	+ 31	− 30.2	57.78	109	140	4.2	18.9	30

* Total balance data for each phase are recorded at the end of each phase, indicated in time as the day from onset of oliguria.

Water exchange

The individual factors which determined the net water exchange (and hence ΔW as calculated in Equation *6* above) were calculated as follows:

"Sensible" water loss (SL) was measured as the sum of water in urine, feces, vomitus, and blood drawn for analysis. "Insensible" water loss (IW) in these patients included water vaporized from lungs and skin, and was taken as equivalent to 91 per cent of the insensible weight loss (IL) as measured (Equation *4*) (9).

Available water included exogenous water administered (H_2O_{ex}) plus endogenous water from oxidation (H_2O_{ox}) of the metabolic mixture and preformed water (H_2O_{pf}) released in the catabolism of tissues. Water of oxidation was calculated (14) as:

$$H_2O_{ox} = 0.6\ C + 1.07\ F + 0.43\ P \qquad (14)$$

and preformed water (pre-existing intracellular water released with cellular catabolism) as:

$$H_2O_{pf} = 18.75\ b_N', \qquad (15)$$

where b_N' is the balance of nitrogen corrected for the change in body fluid urea nitrogen (Equation *2*) and the factor 18.75 is 6.25×3.0 (it being assumed that there are approximately 3 parts of water to 1 part of protein in muscle, the bulk tissue of the body).

The amount of exogenous water in excess of measured "sensible" water loss, which is required to maintain a normal ratio of body water to total body solids, (H_2O_{ex}'), is the difference between the insensible water loss and the amount of water available endogenously:

$$H_2O_{ex}' = IW - (H_2O_{ox} + H_2O_{pf}). \qquad (16)$$

RESULTS

Observed data are recorded in Tables II and III.[5] Chloride balance was uniformly negative during the latter part of oliguria, as was sodium

[5] Complete protocol of representative case is presented in Table VI (Appendix).

balance except in case M. G. In Phases II and III chloride balance was less negative or more positive than sodium balance, a reflection of excessive urinary sodium loss during diuresis, since generally sodium intake exceeded chloride intake. Potassium and nitrogen balances were predominantly negative. Body weight fell an average of 0.27 kilogram per day during Phase I, 0.62 kilogram per day during Phase II, and 0.26 kilogram per day during Phase III. The increase in weight loss during early diuresis correlates well with increasing loss of total body water at this time (see below).

Derived data are presented in Table IV. For the sake of brevity these values are recorded as daily averages and standard deviations for each phase, all individual values having been corrected to a standard body surface area of 1.68 sq. meters (the mean for this group of patients).[6]

A. Metabolic mixture

Caloric expenditure averaged approximately 2,500 calories per day with little variation from patient to patient or from phase to phase. The average amounts of protein, carbohydrate, and fat burned as calculated by the methods described are enumerated. During Phase I fat constituted the major portion of the metabolic mixture and this represents primarily endogenous fat, since little exogenous fat was given. The amount of fat catabolized decreased progressively during Phases

[6] Body surface area in sq. meters $= Wt^{0.425} \times Ht^{0.725} \times 0.007184$ (after DuBois and DuBois [15]).

TABLE IV

Derived data: Mean daily values for metabolic mixture, changes in body composition and exchanges of electrolytes and water*

		Phase†					
		I‡		II		III	
		mean	s.d.	mean	s.d.	mean	s.d.
A. Metabolic mixture							
CHO burned	gm./24 hr.	113	± 18	183	± 42	194	± 28
Protein burned	gm./24 hr.	42	± 19	52	± 21	59	± 15
Fat burned	gm./24 hr.	201	± 32	177	±103	140	± 66
Caloric expenditure	cal./24 hr.	2,405	±150	2,610	±402	2,339	±263
B. Changes in body composition							
ΔFat	gm./24 hr.	−195	± 49	−156	± 89	−110	± 90
ΔW	ml./24 hr.	− 54	±327	−435	±241	− 59	±145
ΔE_{Cl}	ml./24 hr.	− 70	±101	−242	±197	− 33	±119
ΔI	ml./24 hr.	+ 16	±261	−193	±193	− 26	±104
ΔSolids	gm./24 hr.	− 30	± 17	− 64	± 24	− 76	± 36
ΔLBM	gm./24 hr.	− 83	±330	−499	±272	−135	±169
C. Electrolyte exchanges							
ΔCl	mEq./24 hr.	− 16	± 2	− 20	± 28	+ 12	± 22
ΔNa_E	mEq./24 hr.	− 15	± 10	− 30	± 31	− 4	± 17
ΔNa_I	mEq./24 hr.	+ 1	± 9	− 13	± 18	− 12	± 17
ΔK_E	mEq./24 hr.	+ 4	± 7	− 2	± 2	± 0	± 1
ΔK_I	mEq./24 hr.	− 12	± 12	− 18	± 14	− 9	± 10
$\Delta K_I'$	mEq./24 hr.	+ 11	± 8	+ 5	± 11	+ 12	± 12
D. Water exchanges							
Exogenous intake (H_2O_{IN})	ml./24 hr.	1,028	±441	2,780	±470	3,530	±646
Water of oxidation (H_2O_{ox})	ml./24 hr.	303	± 30	332	± 96	291	± 67
Endogenous preformed (H_2O_{pf})	ml./24 hr.	124	± 75	132	± 57	109	± 45
Insensible loss (IW)	ml./24 hr.	981	±141	1,042	±278	954	±206
Sensible loss (SL)	ml./24 hr.	296	±113	2,452	±623	3,056	±579
Water required in excess of SL ($IW - H_2O_{ox} - H_2O_{pf}$)	ml./24 hr.	554	±104	587	±226	554	±156

* Mean of individual values for all patients in each phase corrected to standard body surface area of 1.68 sq. meters (the average body surface area for the group).

† Six patients studied during Phase I, 7 during Phase II, and 6 during Phase III.

‡ Values refer only to latter part of oliguric phase (average 5 days) since studies were not begun at onset of oliguria.

II and III, possibly a reflection of decreasing stores of body fat and/or of increasing exogenous caloric intake. The amount of protein burned averaged approximately 50 grams per day in this series, and the amount of carbohydrate burned was assumed to be equal to that given. (Significant glycosuria was absent in all patients.)

B. Changes in body composition

The derived data from Table IV (B) are presented as *average* cumulative changes in Figure 1, the duration of each phase being the mean for the group. Expressed in this manner, 15 per cent of initial body fat, 14 per cent of initial body water, and 12 per cent of initial body solids were lost during the entire period of study (mean 21 days). Extracellular water was diminished by 25 per cent, and intracellular water by 9 per cent. Expressed in different terms, of the mean loss of body weight 34 per cent represented loss of fat, 52 per cent loss of water, and 14 per cent loss of solids.

Fat was estimated to constitute a relatively large portion of total body weight in these patients, all but one of whom were clinically obese. Body fat was depleted at steadily decreasing rates throughout the period of study. If the rate of endogenous fat catabolism during the early part of Phase I, before studies were initiated, can be assumed to be roughly equal to that calculated during the latter part of this phase (195 grams per day), then mean fat loss during the entire period of oliguria could be adjusted upward to 13 per cent of total fat stores and to 23 per cent during all three phases.

Lean body mass (water and solids) was diminished by an average of 26 per cent during the entire period of study, accounting for approximately 66 per cent of the total weight loss.

Changes in volume of *total body water,* extracellular and intracellular water were variable,

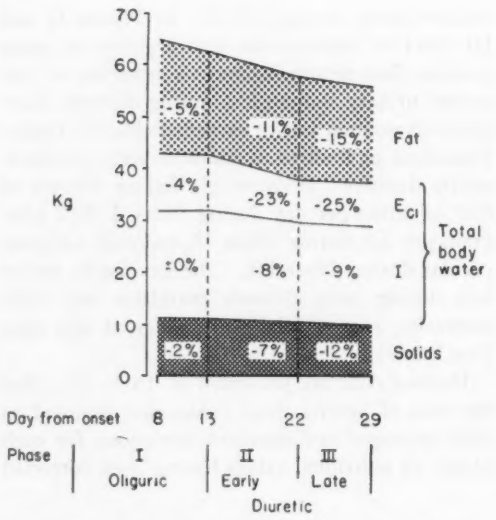

FIG. 1. CHANGES IN BODY COMPOSITION IN ACUTE RENAL FAILURE

Mean cumulative changes in 8 patients of body weight, fat, total water, chloride space (E_{Cl}), non-chloride space (I), and solids, based on the values presented in Table IV (B), and a mean initial body weight of 65 kg.

particularly during Phase I. Mean values, however, indicated a depletion of total body water during each phase, the rate of loss being approximately eight times greater in Phase II (− 435 ml. per day) than in Phases I and III (− 54 and − 59 ml. per day, respectively). Also during Phase II more than 50 per cent of the total water loss came from the chloride space, representing a mean reduction of 19 per cent of this space compared to a mean reduction of 8 per cent of the non-chloride space.

Body solids decreased at slow, gradually increasing rates (30, 64, and 76 grams per day in Phases I, II, and III, respectively). These

TABLE V

Derived data: Estimated mean body composition at start of study and end of Phases I, II, and III, expressed as percentages of total body weight and of lean body mass *

Time	Fat	Water		E_{Cl}		I		Solids	
	% *wt.*	% *wt.*	% *LBM*	% *wt.*	% *LBM*	% *wt.*	% *LBM*	% *wt.*	% *LBM*
Start of study	34.8	48.2	73.8	16.9	25.9	31.3	47.9	17.0	26.2
End Phase I	34.0	48.8	74.0	16.9	25.5	32.0	48.5	17.2	26.0
End Phase II	35.1	47.1	72.5	14.8	22.7	32.3	49.8	17.8	27.5
End Phase III	34.7	47.8	73.2	14.7	22.5	33.1	50.7	17.5	26.8

* For deviation see *Calculations.*

changes, calculated indirectly from changes in body weight minus changes in body fat and water, were in fair agreement with direct measurements of solids lost in urine and feces, averaging 18, 61, and 79 grams per day in successive phases.

While the overall average losses of total body fat, water, and solids were appreciable, relatively little change occurred in body composition in terms of the ratio of each compartment to total body weight (Table V). Thus it would appear that the total decrements of fat, water, and solids were roughly proportional to their respective proportions of the total body weight. This generalization does not hold, however, during the early diuretic phase (Phase II) when total body water loss, particularly extracellular fluid loss, was disproportionately great.

C. Electrolyte changes

The derived data from Table IV (C) are presented as average cumulative balances in Figure

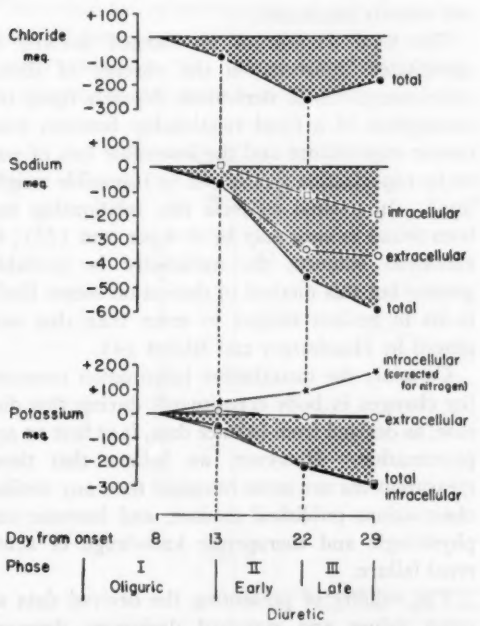

FIG. 2. EXTERNAL BALANCES AND INTERNAL TRANSFERS OF ELECTROLYTES IN ACUTE RENAL FAILURE

Mean cumulative balance of chloride, sodium, and potassium, and mean cumulative changes in intracellular and extracellular sodium and potassium, based on values presented in Table IV (C).

2. Significant mean negative balances of chloride were observed in the first two phases, and of sodium in all three phases. Maximal rates of negative sodium and chloride balance coincided with maximal rates of negative water balance, during the early diuretic phase. Total average negative sodium balance (− 569 mEq.) exceeded total average negative chloride balance (− 181 mEq.) by 388 mEq. Total average potassium balance was − 288 mEq. Maximum negative daily balance of all three electrolytes occurred during Phase II.

Cumulative *chloride* balances were: − 82, − 264, and − 181 mEq. in Phases I, II, and III, respectively. Average daily chloride balances in each of these phases were: − 16.3 (± 2.4), − 20.3 (± 27.7), and + 11.9 (± 21.8) mEq., respectively. The mean positive chloride balance in Phase III associated with a persistent slight mean negative extracellular water balance, accounted for signicant increases in serum chloride concentration in the majority of these patients.

Maximum daily negative *sodium* balance occurred during Phase II (− 43 mEq.) during which time more than twice as much sodium was lost from the chloride space as from the non-chloride space. During Phase III intracellular sodium loss continued at about the same rate (− 12 mEq./day) whereas the rate of extracellular sodium loss decreased appreciably.

Overall mean cumulative *potassium* balances were: − 44, − 233, and − 288 mEq. in progressive phases, respectively. Negative mean daily potassium balance in Phase II (− 19.9 mEq.) was approximately twice that in Phases I and III (− 8.8 and 9.3 mEq.). The less negative balance in Phase III appears to be related more to increased potassium intake (Table II) than to increasing renal conservation. As could be expected, the change in extracellular balance of potassium was minimal and contributed little to the total balance. Mean total intracellular potassium loss was 285 mEq. while total potassium released through cellular catabolism averaged 477 mEq. Thus the difference, 192 mEq., represented the average amount of potassium taken up by remaining intact cells in excess of nitrogen, or the positive intracellular potassium balance corrected for protein burned.

D. Water exchanges

Mean exogenous water intake given in Table IV (D) exceeded mean sensible water loss by 732, 328, and 474 ml. per day in successive phases. Daily water of oxidation averaged 309 ml. and endogenous preformed water averaged 122 ml. with relatively little variation from phase to phase. Insensible water loss averaged 992 ml. per day, also with little variation from phase to phase and from patient to patient.

DISCUSSION

Validity of calculations

These data have been derived by the balance technique and hence yield information concerning changes in, rather than absolute amounts of, certain body constituents. Measurements of absolute amounts of such constituents as body fat, body water, and certain cations were not feasible, and the techniques were not available for use in these patients. However, assessment of total body composition by determination of whole body specific gravity, or by the dilution of isotopically labelled water requires an assumption of the ratio of water to fat-free solids (16–18). While it is extremely desirable to make such measurements in the study of acute renal failure, as has been done in a small number of these patients (6), changes in body constituents as determined by the balance technique are perhaps more readily done, and should provide much useful and more frequent information over shorter intervals of time.

The estimation of lean body mass at the end of the recovery period from the creatinine excretion must be only an approximation. But error in the absolute magnitude of this fraction of the body, or of its aqueous subdivisions, does not invalidate the changes calculated backwards in time from the balance data; only the percentage values would be affected. The calculation should still give a true picture of changes of the principal body fractions in relation to each other.

The use of the chloride space will not be discussed here in detail; this has been done elsewhere in recent publications (13, 19, 20). It should be enough to point out that although the chloride space includes the connective tissue subphase (which the inulin space does not) and any other cellular or transcellular phases penetrated by chloride, this space still essentially excludes the major intracellular fluids of the body, such as those of skeletal muscle. In addition, the spurious steady expansion of the inulin space in nephrectomized dogs and in anuric subjects (21) hardly recommends the use of this measurement over that of the chloride space.

The patients when studied during the oliguric phase presented one particular difficulty in the determination of balance data, namely, that the substances ordinarily excreted in urine are retained in the body fluids. Calculation of the decrement of metabolized protein and potassium in a closed system requires measurement or assumption of the fluid volume through which the concentration is raised. This is less accurate than the direct measurement of nitrogen and potassium excreted. In our calculation of the decrement of nitrogen this involved estimation of the volume of total body water. By use of a series of approximations error in assumption can certainly be minimized though not entirely eradicated.

The total amount of fat burned likewise is quantitated indirectly in the absence of direct calorimetry. Such derivation depends upon the assumption of a fixed relationship between total caloric expenditure and the insensible loss of water by vaporization (measured by insensible weight loss). In normal subjects this relationship has been found to vary only by $\pm$ 4 per cent (*22*); in abnormal subjects the variability is probably greater but this method of derivation seems likely to us to be less subject to error than that employed by Hamburger and Richet (4).

Certainly the quantitative information concerning changes in body constituents during this disease, as derived from balance data, is at best an approximation. However, we believe that these measurements are more complete than any similar observations published to date, and increase our physiologic and therapeutic knowledge of acute renal failure.

The validity of presenting the derived data as mean values and standard deviations deserves some comment. These means were calculated on the basis of individual daily averages in each patient for each phase without weighting the individual averages according to duration of individual studies. However, duplicate calculations weighted

on the basis of patient-days gave mean values almost identical to those presented.

The division of early and late diuretic phases according to time of maximal diuresis is arbitrary and may not correlate precisely with changing phases of renal recovery. It is further recognized that therapy may determine in part the point of maximal diuresis. This criterion was chosen, however, because it is easily recognized clinically and because it coincides with a change in trend of alteration of body composition in the patients studied.

Catabolic response

The catabolic response which is of paramount importance in acute renal failure has been discussed by Swan and Merrill in their excellent and comprehensive review of the clinical course of this disease (23). In two cases reported by these authors, daily protein consumption was estimated at 48 and 22 grams, respectively. Hamburger and Richet calculated an average rate of protein catabolism of 75 grams per day in one case, as estimated by urea production (4). These values are in general agreement with those reported here.

Among the factors which may significantly accelerate protein catabolism in acute renal failure are infection, tissue necrosis, and stress of acute illness (24–26). Another possible determinant of catabolic response is the state of nutrition at the onset of oliguria, catabolic rate being probably greater in the previously well-nourished patients than in those previously debilitated by chronic disease (23). While the present series is too small to assess these various factors quantitatively, it is interesting to note that the maximum rate of protein catabolism was observed in Patient B. S., who had the most severe infection in this group, and that the minimum rate was observed in Patient I. T., the oldest in the group and the only one who had pre-existing debilitating disease (carcinoma).

The demonstration in uremic animals and in man that endogenous protein consumption can be reduced by high intake of non-protein calories has led to the general acceptance of such dietary therapy in acute renal failure (27–29). Certain theoretical and practical advantages of carbohydrate over fat in this regard have been emphasized (6, 30–32). It has also been shown, however, that this protein-sparing effect is inhibited by the presence of extensive tissue damage or severe infec-

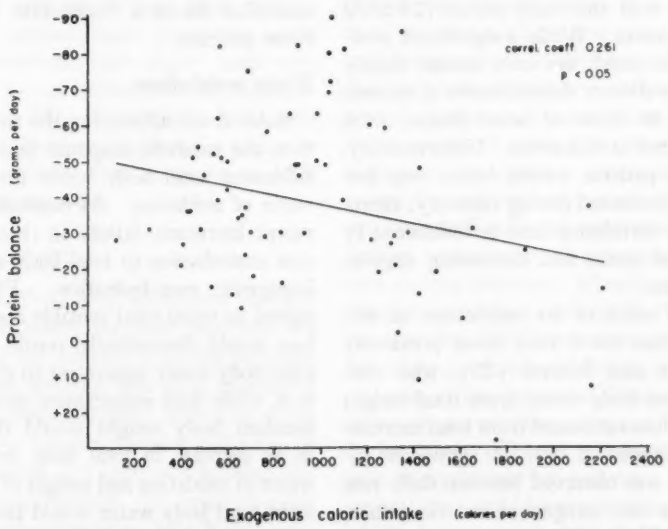

FIG. 3. RELATIONSHIP BETWEEN PROTEIN BALANCE AND TOTAL EXOGENOUS CALORIC INTAKE DURING ACUTE RENAL FAILURE

Points represent average daily values in each patient for each unit balance period (1 to 3 days).

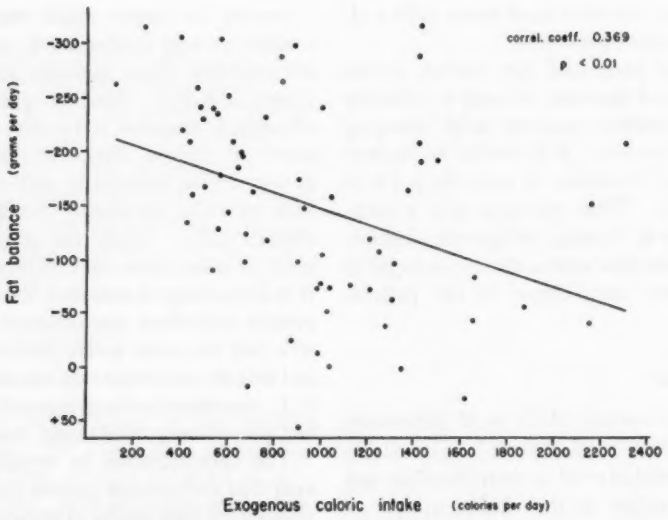

FIG. 4. RELATIONSHIP BETWEEN FAT BALANCE AND TOTAL EXOGENOUS CALORIC INTAKE DURING ACUTE RENAL FAILURE

Points represent daily average values in each patient for each unit balance period (1 to 3 days).

tion (33). An attempt was made in the present series to assess the relationship between protein balance and exogenous caloric intake, largely carbohydrate, in Figure 3, each point representing a daily average for each unit study period (24 to 72 hours) in each patient. While a significant positive correlation is noted, the wide scatter clearly indicates that non-dietary determinants of protein catabolism, such as stress of acute illness, were of major importance in this series. Unfortunately, in most of these patients caloric intake was low initially and was increased during recovery; therefore the apparent correlation may be influenced by improving clinical status and decreasing degrees of stress with time.

The estimated rates of fat catabolism in this series are somewhat lower than those previously derived by Swan and Merrill (23), who subtracted loss of lean body tissue from total weight loss (lean tissue loss estimated from total increase in blood urea nitrogen × 28). A significant inverse correlation was observed between daily rate of fat catabolism and exogenous caloric intake, $r = 0.369$, $p < 0.01$, in Figure 4, but again the above-mentioned non-caloric factors must be taken into account. While the mean rate of fat catabolism decreased with decreasing mean stores of body fat in successive phases, no correlation was observed between individual rates of fat catabolism and initial estimated stores of body fat, *i.e.*, the more obese patients in this series did not catabolize fat at a faster rate than did the less obese patients.

Water metabolism

Aside from influencing the rate of solute retention, the catabolic response in acute renal failure influences total body water through endogenous water of oxidation. As emphasized repeatedly in recent literature, failure to recognize this significant contribution to total body water may lead to iatrogenic over-hydration. Fluid therapy designed to equal total sensible and insensible water loss would theoretically result in an increase in total body water equivalent to the water of oxidation, while fluid replacement designed to maintain constant body weight would theoretically result in an increase in total body water equivalent to water of oxidation and weight of fat burned. Constant total body water would theoretically then be maintained by replacing measured and insensible water loss minus water of oxidation; however, with gradual loss of body solids or lean dry tissue, even such replacement would lead to a gradual

increasing *ratio* of water to body solids. Assuming this ratio to be the primary determinant of the state of hydration, optimal water replacement would therefore be designed to approximate total sensible and insensible water losses minus water of oxidation *and* preformed water released through cellular catabolism ($SL + IW - H_2O_{ox} - H_2O_{pf}$).

In the present series, during the oliguric phase, daily insensible water loss averaged 981 ± 141 ml., a value somewhat higher than those reported in two oliguric patients as determined from water turnover rates using D_2O (34). The validity of the latter method, however, depends upon maintenance of constant total body water, which was not ascertained in the cases reported. Water of oxidation during oliguria in our patients averaged 303 ± 30 ml. and preformed water averaged 124 ± 75 ml. daily. On the basis of these mean values a constant ratio of total body water to body solids would have required approximately 550 ml., or 330 ml. per sq. meter of body surface area, per day, in excess of measured water loss.

This value serves as a useful guide for fluid replacement in most oliguric adults. It may, of course, have to be revised upward or downward according to individual circumstances, particularly according to the initial state of hydration of the patient. Previous estimates of basic fluid requirements in oliguric patients, based largely on water exchanges in normal subjects under basal conditions, were in close agreement with the value reported here (6, 35, 36). Neither the latter estimates nor our own, however, support the arbitrary recommendations of daily fluid intake up to 1500 ml. in oliguric patients (37).

Fluid and electrolyte changes

With regard to fluid and electrolyte changes during the oliguric phase, the data presented are too incomplete and too variable to justify generalizations. The most striking and most consistent changes in fluid volume, however, occurred in the early diuretic phase when a marked reduction in mean total body water, and especially in mean extracellular fluid, was observed. In terms of mean ratios of these compartments to lean body mass, however, (Table V), it is equally significant that the percentage of total body water fell only 1.5 per cent and that the percentage of intracellular fluid actually increased 1.3 per cent, while that of extracellular fluid decreased 2.8 per cent. In this sense, then, one can interpret the predominant volume changes during early diuresis as a *relative* expansion of intracellular fluid and a *relative* contraction of extracellular fluid. Since of necessity these studies were not initiated at the onset of oliguria, one can only speculate as to the fluid alterations which occurred during early oliguria. However, if the changes observed during diuresis can be interpreted, at least qualitatively, as compensatory adjustments to previous alterations in the opposite direction, they would be compatible with the supposition of a previous overexpansion of extracellular fluid and relative contraction of intracellular fluid volume during early oliguria. This interpretation would be in keeping with the observations of others (1–3, 6) and would gain some support from the fact that adequate fluid restrictions were not imposed in our patients until the time of transfer to our service.

Likewise, the greatest electrolyte changes were noted in Phase II, and consisted of a marked negative balance of sodium, and to a lesser degree, of chloride and potassium. The disproportionately excessive loss of sodium throughout diuresis may be attributable in part to accelerated loss of retained non-chloride anions at a time of impaired renal tubular conservation of cations. Mean intracellular sodium balance remained steadily negative throughout both the early and late diuretic phases, even though extracellular sodium appeared to be conserved during the latter phase. Simultaneously intracellular potassium balance (corrected for cellular catabolism) remained positive to a comparable degree, suggesting a roughly quantitative replacement of potassium for sodium in cells during diuresis. Muscle biopsies were not obtained to confirm a rising K–N ratio (corrected for changing NPN) nor were intracellular transfers of magnesium or hydrogen ion calculated.

Again, if one considers these shifts as compensatory in nature, the inference is suggested that during early oliguria potassium may have left cells in exchange for sodium. Support for this supposition would be gained from the experiments which demonstrated the movement of potassium out of cells in acutely uremic dogs with acidosis (38). Similarly, in the balance experiments of Elkinton, Tarail, and Peters (39), potassium left

TABLE VI

Appendix: Complete protocol of observed data in one case (V. N.)

Study periods (days from onset of oliguria)	Phase	Body weight	Serum concentrations						Intake (per 48-hr. period)							
			Cl	Na	K	CO_2	Creatinine	Blood urea nitrogen	H_2O	Solids	Cl	Na	K	N	CHO	Fat
		kg.	*mEq. per L.*	*mEq. per L.*	*mEq. per L.*	*mM. per L.*	*mg. per 100 ml.*	*mg. per 100 ml.*	*gm.*	*gm.*	*mEq.*	*mEq.*	*mEq.*	*gm.*	*gm.*	*gm.*
8	I	79.43	97	139	5.1	28.7	8.6	81								
10		79.89	91	137	5.1	25.2	11.4	92	2,581	333	0	0	0	0	331	0
12		79.08	92	133	5.2	21.8	13.2	118	2,532	239	0	3	4	0.3	172	78
14	II	78.59	88	132	5.5	20.2	14.4	150	3,178	369	53	113	21	0	359	0
16		77.26	95	135	5.7	20.8	13.6	168	3,985	429	264	288	56	0	406	0
18		74.33	100	137	4.9	21.8	10.3	168	5,148	501	347	367	60	0	481	0
20		72.48	91	138	4.0	25.0	9.7	148	6,480	541	460	477	54	0	482	0
22	III	70.20	103	140	4.0	23.8	6.8	90	5,283	490	420	379	93	5.0	463	2
24		69.28	101	140	3.6	25.4	4.4	80	6,099	625	314	338	102	2.0	551	28
26		67.99	99	139	3.6	26.9	2.9	54	5,456	545	230	232	115	8.0	414	55
28		67.94	107	140	4.0	27.6	1.9	42	6,352	466	328	332	159	6.0	320	53
30		68.71	98	138	4.0	30.3	1.7	20	6,823	603	245	249	201	16.0	509	72
32		68.50	100	139	4.0	31.0	1.2	11	6,060	668	219	224	223	17.0	458	121

Study periods (days from onset of oliguria)	Phase	Output (per 48-hr. period)												
		Urine							Stools, *etc.*					
		Vol.	Specific gravity	Cl	Na	K	N	Creatinine	H_2O	Solids	Cl	Na	K	N
		ml.		*mEq.*	*mEq.*	*mEq.*	*gm.*	*mg.*	*ml.*	*gm.*	*mEq.*	*mEq.*	*mEq.*	*gm.*
8	I													
10		315	1.012	21	24	9	1.2	216	132	11	15	10	0	1.5
12		775	1.009	46	57	17	3.3	465	242	11	27	18	0	1.8
14	II	1,860	1.007	104	138	27	7.2	856	274	27	23	15	1	1.2
16		4,130	1.008	256	310	68	15.1	1,404	532	128	15	10	0	1.1
18		6,610	1.010	452	551	139	26.7	2,195	160	11	15	10	0	1.1
20		7,320	1.009	506	579	169	34.3	2,657	89	2	9	6	0	0.7
22	III	6,900	1.009	458	488	176	35.9	2,560	261	31	11	10	35	2.2
24		5,630	1.009	349	390	153	33.7	2,168	277	—	19	14	5	0.4
26		5,180	1.009	296	324	137	32.3	1,948	112	—	9	3	0	0.2
28		4,750	1.007	260	265	135	27.6	1,596	721	90	19	29	27	6.2
30		4,950	1.006	284	231	142	21.4	1,584	299	38	8	13	26	2.6
32		3,840	1.005	160	128	120	10.0	1,344	1,062	173	22	31	93	6.3

cells in excess of nitrogen in 4 of the 6 oliguric patients studied; however, cellular sodium transfers were variable in these 4 patients.

SUMMARY

From balance studies carried out in 8 female patients during the oliguric phase, the early diuretic phase, and the late diuretic phase of acute renal failure, mean values were derived regarding the metabolic mixture, changes in body composition, and exchanges of water and electrolytes.

1. With an average expenditure of 2,500 calories per day, endogenous fat constituted the main source of energy, particularly during oliguria. The mean rate of endogenous protein catabolism remained relatively constant from phase to phase in all patients. The daily rate of endogenous protein catabolism appeared to be only slightly influenced by variations in total exogenous caloric intake, suggesting that non-dietary determinants of catabolic response played a more important role.

2. Mean rates of loss of body fat, water, and solids were roughly proportional to the mean ratios of these compartments to total body weight, except during early diuresis; in this phase a disproportionately great loss of extracellular fluid (chloride space) was observed in all patients.

3. Mean sodium balances were negative in all phases studied, maximally so during early diuresis. Throughout the early and late diuretic phases mean sodium balance remained significantly more negative than mean chloride balance. This was attributable in part to defective tubular conservation of cations at a time of accelerated excretion of retained non-chloride anions. Diuresis was also attended by a mean intracellular shift of potassium in exchange for sodium in approximately equal amounts.

4. By subtracting mean values for water of oxidation and preformed water released through cellular catabolism from mean values of insensible water loss, an estimate was made of the amount of exogenous water required in excess of measured water loss, to maintain a constant ratio of total body water to total body solids. This value averaged 550 ml. per day or 330 ml. per sq. meter of body surface area per day, during oliguria. The therapeutic implications of this finding were discussed.

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INTERRELATIONSHIPS OF SOME METABOLIC EFFECTS OF TESTOSTERONE PROPIONATE IN NORMAL MALES DURING RESTRICTED SODIUM INTAKE [1, 2]

By E. RAYMOND BORUN [3] and ERNEST GEIGER with the technical assistance of LOTTA DALBERG

(From the Departments of Medicine and Pharmacology, University of Southern California School of Medicine, Los Angeles, Calif.)

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Testosterone propionate (T.P.) has been used to stimulate protein anabolism in a variety of clinical conditions (1–5). Metabolic studies have repeatedly demonstrated that the hormone produces nitrogen retention and an increase in body weight in normal subjects as well as in hypogonad individuals (1–6). However, there are discrepancies between the amount of weight gained and the amount of tissue synthesis calculated from the nitrogen retention (1–4, 6). Usually the weight gain is greater than the calculated tissue synthesis but occasionally considerable nitrogen retention occurs without a parallel increase in body weight (7). The weight gain in excess of calculated tissue synthesis has been attributed to an accumulation of extracellular fluid secondary to sodium and chloride retention (1–3); in some instances retention of these electrolytes is indeed sufficient to account for the major portion of the weight gain as an isosmotic increment of extracellular fluid (1–3). Such observations suggest that the relationship between the change in body weight and nitrogen retention might be clarified if sodium and chloride retention were rigidly controlled. We therefore studied the metabolic effects of the hormone in normal adult males maintained on restricted sodium and chloride intake.

PROCEDURE

Subjects and experimental plan: Six well-nourished normal young adult males (five medical students and one physician) were subjects for this study. Routine medical histories, physical examinations, blood counts, and urinalyses revealed no significant abnormalities. Other data are given in Table I. The subjects continued their normal activities during the study but were requested to avoid physical exertion or environmental situations which might result in sweating or chilling. Despite the fact that the experiments were performed in the winter and spring, sensible perspiration could not be entirely eliminated. The five medical students lived together and underwent the experiments simultaneously; the study of one subject (E.R.) preceded this and differed in some details.

Experiment A consisted of a four-day preliminary adjustment to the special diet, a ten-day control period, and ten days of T.P.[4] administration (100 mgm. in oil I.M. daily). Experiment B consisted of a four-day preliminary period, ten days of T.P. administration in the same dosage, and a ten-day recovery period. In three subjects (M. L., S. C., and S. M.) experiment B followed A after a six-week rest period; in the other subjects experiment B was performed first and was followed by A after the six-week rest period. In one subject (E. R.) the preliminary periods were of two days duration and the experiments were terminated after twenty days.

In four subjects (E. R., R. B., S. C., and S. M.) the ability to excrete a standard water load was tested at similar intervals during each ten-day period. The water load (20 ml. per Kg. body weight) was ingested within 10 to 15 minutes and urine specimens were voided every half hour for four hours. The water was ingested about 8:00 A.M. in the fasting state and the subjects remained in bed during the next four hours except while voiding; breakfast and lunch were eaten at the conclusion of the experiment.

Diet: The quantities of food and fluid were self-selected prior to the study from a limited list which provided a low sodium diet (12 to 16 mEq.) adequate in all essential constituents. Dietary intake (Table I) remained essentially constant throughout all metabolic periods. Fluid intake was constant except for the additional amounts used in the diuresis experiments and a few other exceptions (Table II [5]). The diets of all subjects except

[1] Supported by a grant from the Los Angeles County Heart Association.

[2] Some of the data were previously published in Clin. Res. Proc., 1955, 3, 60.

[3] This study was done during the tenure of an Orange County Heart Association research fellowship. Present Address: Department of Medicine, University of California Medical Center at Los Angeles, Los Angeles 24, California.

[4] Supplied by the Schering Corporation.

[5] Table II (body weight and metabolic balance data) has been deleted at the suggestion of the editors. Copies of the table are available on request.

TABLE I

Clinical and dietetic data

Subject	Age	Height *cm.*	Weight *Kg.*	Daily Dietary Intake: Protein *Gm.*	Carbohydrate* *Gm.*	Fat* *Gm.*	Calories*
F. R.	26	171	66.50	112	320	142	3,089
R. B.	23	183	77.75	104	265	112	2,554
S. C.	24	180	70.50	105	296	112	2,685
S. M.	23	187	86.05	121	368	141	3,319
E. R.	35	178	70.75	88	223	101	2,209
M. L.	26	192	81.70	119	362	128	3,155

* Calculated from food tables (11).

E. R. were high in protein (105 to 125 Gm.) and potassium (150 to 165 mEq.) to provide optimal conditions for protein synthesis. These characteristics were obtained in a palatable and easily prepared diet by using liberal quantities of a fresh low-sodium milk.[6] One subject (M. L.) added 3 Gm. of sodium chloride to his daily diet to provide a comparison between the effects of T.P. during moderate and low sodium intake in the same experimental situation. Single lots of canned and prepared foods were used when feasible. Lean ground round steak was weighed out in individual portions and stored frozen; portions were weighed again after cooking to determine average fluid loss. All other foods were weighed and fluids measured after preparation. Distilled water was used throughout the study.

Observations and collections of specimens: Body weight was measured to the nearest ⅛th pound every morning after voiding (weights were converted to the nearest 0.050 Kg.). Twenty-four hour urine volume and specific gravity were measured; the specimens were acidified with sulfuric acid and toluene was added as a preservative. Stools were weighed daily, homogenized in 4 or 5 day pools, acidified, and stored frozen. Half portions of a complete diet were homogenized daily, acidified, and stored frozen. Fasting blood specimens were drawn after removal of the tourniquet; the portion for electrolyte analysis was immediately placed under oil and the serum promptly removed after clotting had occurred.

Analytical methods: Dry weights of diets, stools, and pooled urine aliquots, were determined by drying 2 to 5 Gm. portions to constant weight in a vacuum dessicator at 75° C. Diet and stool homogenates and two-day urine aliquots were analyzed for total nitrogen by semi-micro and macro-Kjeldahl methods, sodium and potassium by Perkin-Elmer flame photometer with lithium internal standard, and chloride by the Volhard-Arnold method (8). Diet and stool homogenates were prepared for electrolyte analysis by digestion with nitric acid (9). The chloride concentration of diet and stool digests were below the range of optimum accuracy for the Volhard-Arnold method. Serum nitrogen, sodium, and potassium, were determined by the same methods, chlorides by the method of Schales and Schales (10), and NPN by nesslerization. Nitrogen and chloride determinations were done in duplicate, and urine nitrogen determinations were repeated in duplicate.

Calculations: Fat and carbohydrate content of the diets were calculated from food tables (11); values for the other constituents in Table I were obtained by averaging analytical results from eight aliquots of each diet. Nitrogen, potassium, sodium and chloride balances were calculated for five-day periods by subtracting the amounts excreted in the urine and stool from the intake. Since the urines of experimental days 5–6 and 15–16 were analyzed as single two-day aliquots, half the total values from these aliquots were arbitrarily assigned to the appropriate five-day period.

Water balance was calculated from the formulas of Newburgh and his associates (12, 13) and Lavietes (14): Δ Water $= \Delta$ Weight $+ (S_e - S_i) + C_i + F_b + 3.06\ N_u$ where S_e is solids excreted, S_i is solids ingested, C_i is carbohydrates ingested, F_b is fat burned, and N_u is urine nitrogen, all in grams. The fat burned was calculated from the formula of Newburgh, Johnston, Lashmet, and Sheldon (13): $F_b = \dfrac{IL - (2.18\ C_i + 12.26\ N_u)}{3.93}$, where IL is insensible weight loss and is calculated from the formula: $IL = (W_i - W_f) + (W_t - W_e)$, where W_i, W_f, W_t, and W_e are, respectively, the initial and final body weights and weights of ingesta and excreta. The calculations are subject to errors inherent in the basic assumptions and technique as well as errors due to sensible perspiration and fluctuations of body temperature (14). The water balance data are presented and interpreted with these reservations in mind.

The mean changes in body weight, and the water, nitrogen, potassium, sodium, and chloride balances during the preliminary and control periods were determined in the group of subjects on low sodium intake. Differences between control period data and similar data obtained during the T.P. and recovery periods in the same subject were used to calculate "net" changes (Tables II[6] and III). Differences between the two entire T.P. periods and also between the corresponding halves of each T.P. period were determined by comparing both the means and the individual differences. Similarly, changes that occurred during the first and second half of every period were compared with each other and with the entire control period. Ratios between two different types of data

[6] Supplied by Edgemar Farms, Venice, California.

TABLE III

Mean net changes in body weight and balance data during and after T.P. administration in five subjects on low sodium intake

	Body weight			Water			Nitrogen			Potassium			Sodium			Chloride		
	*Gm.**	S.E.†	P‡	*Gm.**	S.E.	P	*Gm.**	S.E.	P	*mEq.**	S.E.	P	*mEq.**	S.E.	P	*mEq.**	S.E.	P
T.P.—First 5 days	+845	85	<.01	+860	73	<.01	+ 8.4	0.81	<.01	+88	9.9	<.01	+ 7	3.7	<.1	+ 5	9.4	>.5
T.P.—Second 5 days	+485	126	<.01	+500	89	<.01	+15.0	1.78	<.01	+93	16.6	<.01	+18	2.6	<.01	+27	8.1	<.01
Recovery—First 5 days	−545	189	<.05	−415	57	<.01	+10.3	1.62	<.01	+29	22.7	<.3	+18	3.9	<.01	+22	9.5	<.05
Recovery—Second 5 days	−480	77	<.01	−340	156	<.2	+ 2.1	1.66	<.3	−69	20.5	<.05	+10	3.3	<.05	+ 5	11.9	

* Means of the individual net balances (Table II)[6] calculated by subtracting each subject's average control period data from experimental period data.
† Standard error of the mean (15).
‡ P value for the "t" test (15).

were calculated as the quotients of the mean net changes (Table IV). The Fisher "t" test was applied to the standard errors of the mean changes, differences between means, means of individual differences, and the ratios of the means (15). The degrees of freedom were assumed to be one less than the number of values used in each calculation. Since two sets of T.P. period values were obtained from each subject, this was not a true random sample and therefore the statistics must be used with caution for generalizations beyond the present group of subjects. However, when the two sets of T.P. data from each subject were averaged and the degrees of freedom were assumed to be one less than the number of subjects, the level of significance for mean net changes and means of individual differences between the two halves of the T.P. periods were similar to those obtained from the previous type of calculations. Differences are considered significant with $p < 0.01$ and probably significant with $p < 0.05$.

RESULTS

Systemic and local reactions

The control periods were uneventful. There were no complaints about the monotony or palatability of the food even after 24 days on the constant low-sodium diet. Four subjects noted sensations of increased temperature, perspiration unrelated to exertion, nervousness, or tachycardia, during T.P. administration. Two subjects had temperatures of 99.6° F, 100.2° F and 100.6° F on single occasions coinciding with the sensation of feverishness. Pulse rates were not determined. One subject (S.C.) had an acute, non-febrile pharyngitis and laryngitis during the initial four days of T.P. in Experiment B. All subjects noticed local discomfort and tenderness after the T.P. injections, varying from mild to moderately severe and lasting 24 to 48 hours.

Metabolic Observations in the Five Subjects on Low Sodium Intake

Body weight and water balance

Body weight and water were lost during the preliminary periods on the diet (1,160 ± 139 Gm.[7] and 940 ± 136 Gm. respectively). Variable changes occurred in weight and water balance during the control period; the differences between the changes in weight and water balance should theoretically depend on discrepancies between the in-

[7] Figures presented in this manner represent the mean ± standard error.

TABLE IV

Ratios between mean net changes in body weight and balance data during T.P. administration in five subjects on low sodium intake

Period	Gm. Wt./Gm. N	Gm. H_2O/Gm. N	Gm. Wt./mEq. K	Gm. H_2O/mEq. K	mEq. K/Gm. N
T.P.—First 5 days	101 ± 10.1*	102 ± 8.7	9.6 ± 0.96	9.8 ± 0.83	10.5 ± 1.18
T.P.—Second 5 days	32 ± 8.4	33 ± 5.9	5.2 ± 1.36	5.4 ± 0.96	6.2 ± 1.07

* Quotient of the mean net changes (Table III) ± standard error (15). These mean net changes are all positive, indicating an increase in body weight, water, nitrogen, and potassium during the T.P. periods.

take and expenditure of calories during that period (12–14).

All five subjects had net weight gain and net water retention during both T.P. periods as compared with the control period; weight gain was greater in Experiment A than Experiment B ($p < 0.05$). The mean net weight gain (1,280 ± 161 Gm.) and water retention (1,315 ± 162 Gm.) were significant, and the larger portion of both changes occurred during the first five days of T.P. ($p < 0.02$). The mean net water retention/weight gain ratio during T.P. administration was 1.02 ± 0.18.

There was net weight loss (1,100 ± 298 Gm.) and water loss (740 ± 204 Gm.) in all five subjects during the recovery period. The net water loss was considerably less than weight loss in two subjects and more than the weight loss in the others.

Nitrogen balance

During the control period the nitrogen balance was negative in two subjects who lost weight and was positive in the three who gained weight. Net nitrogen retention occurred in all five subjects during both T.P. periods, and was greater in Experiment A than in Experiment B ($p < 0.05$). The mean net nitrogen retention during T.P. periods (23.1 ± 2.4 Gm.)[8] was significant, and the larger portion ($p < 0.01$) occurred during the second five days. The amount of nitrogen which each subject retained during T.P. periods was not related to his control period balance.

The ratios of net weight gain/nitrogen retention and of water retention/nitrogen retention during the first five days of T.P. administration (101 ± 10 and 102 ± 8, respectively) were both significantly greater than the ratios during the second five days (32 ± 8 and 33 ± 6, respectively).

Net nitrogen retention continued during the recovery period in all five subjects (11.0 ± 1.6 Gm., $p < 0.05$); however, the significant retention was limited to the first five days of the period (10.3 ± 1.6 Gm.).

Potassium balance

The potassium balance was negative in all the control periods (109 ± 51 mEq.) despite the high dietary intake, and did not correlate with the simultaneous nitrogen balance. Since the control period potassium balance in the subject receiving a sodium chloride supplement was negative also, this could represent an undetected systematic error rather than a secondary effect of sodium restriction (17).

Net potassium retention occurred in all five subjects during the T.P. periods (181 ± 25 mEq.). There was no significant difference between the two T.P. periods or between the first and second halves of these periods. The ratios of net weight gain/potassium retention and of water retention/potassium retention during the first five days of T.P. (9.6 ± 1.0 and 9.8 ± 0.8, respectively) were significantly greater than during the second five days (5.2 ± 1.4 and 5.4 ± 1.0, respectively). The ratio of net potassium retention/nitrogen retention during the first five days of T.P. (10.5 ± 1.2) was significantly greater than during the second five days (6.2 ± 1.1).

Changes in potassium balance were not consistent during the first half of the recovery period, but there was significant net loss during the second half (69 ± 21 mEq.).

Sodium and chloride balance

Sodium and chloride were lost during the preliminary period on the diet (165 ± 38 mEq. and

[8] Correction of the mean net nitrogen balance for changes in NPN concentration (16) does not alter this figure significantly.

188 ± 21 mEq. respectively). The control period sodium balances were slightly positive (64 ± 16 mEq.) but correction for skin loss would reduce these figures considerably (18). Net changes in sodium and chloride balances were not significant during the first five days of the T.P. periods. Very slight but statistically significant net sodium and chloride retention occurred during the second five days of the T.P. periods (18 ± 3 mEq. and 27 ± 8 mEq. respectively), and continued during the recovery period (24 ± 5 mEq. and 27 ± 20 mEq.).

Metabolic Observations in the Subject Receiving a Sodium Chloride Supplement (M. L.)

Despite the 51 mEq. of sodium chloride which this subject added to his food daily, he lost weight and was in negative water, sodium, and chloride

TABLE V

Blood chemistry

Subject	Exp.	Period	Day	Na* *mEq./L.*	Cl* *mEq./L.*	NPN *mgm. %*	Protein* *Gm. %*
F. R.	B	T.P.	5		103.0	36	7.40
		Recovery	15		102.2	35	6.85
			25		101.3	30	7.09
	A	Preliminary	1	135.6	106.6	30	6.41
		Control	5	131.0	101.7	44	6.97
		T.P.	15	130.4	102.1	36	7.03
			25	130.0	100.4	37	6.85
R. B.	B	T.P.	5		98.3	38	8.23
		Recovery	15		96.9	36	7.26
			25		98.7	33	7.28
	A	Preliminary	1	136.7	103.8	38	7.27
		Control	5	135.8	101.3		7.68
		T.P.	15	136.7	101.1		7.88
			25	135.0	97.9		7.36
S. C.	A	Control	5			35	7.11
		T.P.	15		100.9	34	7.21
			25		98.7	28	7.08
	B	Preliminary	1	136.8	103.4	34	7.04
		T.P.	5	135.6	103.5	40	7.23
		Recovery	15	134.2	99.4	37	7.05
			25	133.7	101.0	40	6.98
S. M.	A	Control	5		106.4	37	7.55
		T.P.	15		100.9	37	7.03
			25		98.7	31	6.99
	B	Preliminary	1	137.0	104.0	37	6.84
		T.P.	5	136.7	99.8	43	6.67
		Recovery	15	133.7	97.9		6.91
			25	135.0	102.3	33	6.77
E. R.	B	T.P.	3	132.6			
		Recovery	13	133.5			
			18	130.4			
	A	Control	3	134.7			
		T.P.	13	136.8			
M. L.	A	Control	5		105.6	38	7.20
		T.P.	15		100.9	38	6.98
			25		101.7	29	7.15
	B	Preliminary	1	137.0	104.9	36	6.80
		T.P.	5	133.0	102.2	43	7.25
		Recovery	15	136.1	101.1	42	6.64
			25	128.4	101.7	39	7.14

* Concentration in serum.

balance during both preliminary periods on the diet. His mean net weight gain (2,030 Gm.) and water retention (2,330 Gm.) during the T.P. periods were greater than the changes in the group on low sodium intake; the significant difference occurred during the second five days of these periods. The mean net nitrogen retention (20.8 Gm.) and potassium retention (222 mEq.) during the T.P. periods were similar to the retention in the low sodium group, while the mean net sodium retention (160 mEq.) and chloride retention (100 mEq.) were significantly greater in this subject. The major retention of nitrogen occurred in the second half of the T.P. periods, as it did in the low sodium group, and both sodium and chloride retention were also greater in the second half of the T.P. periods while potassium retention occurred approximately equally in the two halves.

There was net loss of both weight (1,550 Gm.) and water (950 Gm.) during the recovery period despite the continuation of net nitrogen retention (19.9 Gm.); but unlike the results in the low sodium group, the recovery period changes in this subject were accompanied by net loss of both sodium (132 mEq.) and chloride (124 mEq.).

Blood chemistry (Table V)

The small magnitude of the changes and incompleteness of the blood chemistry data preclude any definite conclusions about the effects of T.P. administration. The subjects on low sodium intake showed slight decreases in plasma protein and chloride concentration during T.P. periods (0.29 ± 0.13 Gm. per cent and 2.1 ± 0.4 mEq. per L., respectively) but when compared with control period data the net effects were not significant.

Water diuresis experiments

A total of nineteen water diuresis experiments were performed in four subjects, as described under Procedures. No consistent differences were observed between results in T.P. periods and control or recovery periods in terms of the time course of diuresis, maximal rate of urine flow, or total volume excreted.

DISCUSSION

In this study the sodium retaining effect of T.P. was controlled in a group of normal adult males by restricting dietary sodium intake; the sodium excretion dropped to such low levels after a few days of diet restriction that the hormone could not cause much further decrease. The experimental design thus minimized one factor that may account for a variable portion of the weight gain during T.P. administration (1–3), and allows one to compare the changes in body weight and water more directly with changes in nitrogen and potassium balance. However, the effects of the dietary regimen itself, which probably include stimulation of aldosterone secretion (19, 20), must be differentiated from changes produced by T.P. Comparison of data from the two T.P. periods with that from the control periods may make this differentiation possible and also compensate to some extent for systematic errors in methodology.

The anticipated major adjustments to the low sodium diet occurred during the preliminary periods (17); T.P. administration was then started on the fifth day of the dietary regimen in one experiment and on the fifteenth day in the other experiment so that delayed effects of the diet (17) might be revealed as differences between the two T.P. periods in each subject. The only significant differences observed between the two T.P. periods were the more marked weight gain and nitrogen retention in the experiment where T.P. was started early. Although these differences may be related to the duration of sodium restriction, the fact that they were also present in data from the subject receiving a sodium chloride supplement suggests that they could be secondary to other factors.

T.P. administration consistently produced a weight gain which was somewhat greater than the amount calculated on the basis of the nitrogen retention, assuming a ratio of 32 Gm. of weight per Gm. of nitrogen (21–26) (Table IV and Figure 1). Our water balance calculations indicate that the weight gain was essentially due to fluid retention. The subjects on low sodium intake showed an increase in body weight and water out of proportion to the relatively small amount of nitrogen and insignificant amounts of sodium and chloride retained during the first five days of T.P. administration. During the second five days of T.P. administration the increments of body weight and water were related to the nitrogen retention

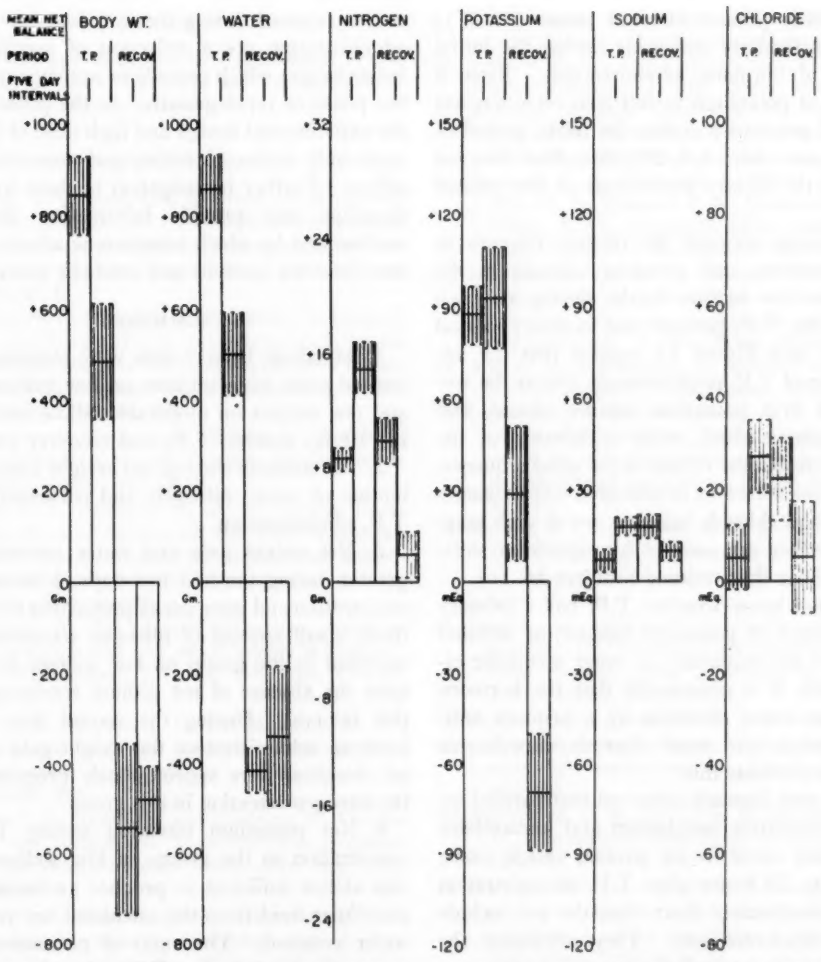

FIG. 1. MEAN NET CHANGES IN BODY WEIGHT AND BALANCE DATA DURING AND AFTER T.P. ADMINISTRATION IN FIVE SUBJECTS ON LOW SODIUM INTAKE

The shaded areas represent the standard error of each mean. The scales for nitrogen and body weight are related according to the ratio in adult tissue, potassium and water according to the ratio in intracellular fluid, and sodium, chloride and water according to their ratios in extracellular fluid.

by ratios resembling those of normal adult tissue. These subjects lost weight and water during the first five days after T. P. was withdrawn despite the fact that nitrogen retention continued during this period along with slight sodium and chloride retention. Since similar disproportions between body weight and nitrogen balance follow T.P. administration and withdrawal during both normal (1–4, 6) and restricted sodium intake, these effects are probably not dependent on the level of sodium intake or sodium retention.

The ratio of water to potassium retained during the first five days of T.P. administration was greater than that normally found in intracellular fluid (*24–27*), but the ratio approximated that of intracellular fluid by the end of the ten-day period. The ratio of potassium to nitrogen retained during T.P. administration was greater than the

ratio normally present in adult tissue (23–27); this was particularly noticeable during the initial five days of hormone administration. Since a high ratio of potassium to nitrogen retention has been noted previously during the initial period of T.P. administration (4, 6, 28), this effect does not depend on the dietary restrictions of the present study.

Comparisons between the relative changes in water, potassium, and nitrogen balances in the subjects on low sodium intake during the two halves of the T.P. periods and recovery period (Table IV and Figure 1) suggest that the administration of T.P. predominately affects the water balance first, potassium balance second, and nitrogen balance third, while withdrawal of the hormone reverses the effects in the same sequence. These relationships may be obscured when changes in sodium and chloride balances are of such magnitude that they can account for significant shifts in extracellular fluid volume (subject M. L.).

We do not know whether T.P. has a primary effect on water or potassium balance, or whether the changes are secondary to other metabolic effects. While it is conceivable that the hormone might cause water retention by a primary antidiuretic action, our water diuresis experiments failed to demonstrate this.

Rudolph and Samuels observed that parallel increases in oxidative metabolism and intracellular water content occur in rat seminal vesicle tissue within 10 to 20 hours after T.P. administration (29). Unfortunately their data do not include potassium determinations. They attributed the initial increase in intracellular water to an accumulation of osmotically active particles accompanying the change in oxidative metabolism. This explanation could not be applied to the present study without modification because total oxygen consumption in man is usually not increased during the first few days of T.P. administration (2, 30).

Relatively high ratios of water to nitrogen and of potassium to nitrogen occur in rapidly growing tissues such as the human fetus (31, 32), regenerating rat liver (33–35),[9] and certain neoplasms (35–37). It is possible that the high ratios of water to nitrogen and of potassium to nitrogen retained during the first few days of T.P. administration are a reflection of specific metabolic changes which precede or accompany the initial phase of rapid growth. In the present study the experimental design and high dose of hormone apparently tended to isolate and accentuate these effects. Further investigation of these inter-relationships may provide information about the mechanisms by which testosterone affects the balance between anabolic and catabolic processes.

[9] Two studies describe separately a decrease in nitrogen (34) and an increase in potassium (35) per unit dry weight of regenerating rat liver.

SUMMARY

1. Metabolic balance data were obtained in five normal adult male subjects on low sodium intake and one subject on moderate sodium intake during 10-day control, T.P., and recovery periods.

2. All subjects showed net weight gain and retention of water, nitrogen, and potassium during T.P. administration.

3. Net weight gain and water retention were greater during the first five days of hormone administration and were out of proportion to the relatively small amount of nitrogen retention. This occurred in the group on low sodium intake despite the absence of net sodium retention during this interval. During the second five days of hormone administration the weight gain and water retention were approximately proportional to the nitrogen retention in this group.

4. Net potassium retention during T.P. administration in the group on low sodium intake was almost sufficient to produce an isosmotic intracellular fluid from the calculated net volume of water retained. The ratio of potassium to nitrogen retained during T.P. administration was considerably higher than that found in normal adult tissue; this ratio was highest during the initial five days of hormone administration.

5. All subjects lost weight and water during the first five days after T.P. was withdrawn despite the fact that the net nitrogen balance was still positive, and the net sodium and chloride balances were also slightly positive in the group on low sodium intake.

6. The results suggest that in the absence of significant sodium and chloride retention T.P. affects the water balance first, potassium balance second, and nitrogen balance third, while withdrawal of the hormone reverses the effects in the same sequence.

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EXPERIENCES WITH RADIOSULFATE IN THE ESTIMATION OF PHYSIOLOGIC EXTRACELLULAR WATER IN HEALTHY AND ABNORMAL MAN [1, 2]

By ROBERT J. RYAN, LUKE R. PASCAL, TOHRU INOYE, AND LIONEL BERNSTEIN

(From the Department of Medicine, University of Illinois, Chicago, Ill., and the U. S. Army Medical Nutrition Laboratory, Fitzsimmons Army Hospital, Denver, Colo.)

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We have been interested in studying the changes in extracellular fluid volume in patients undergoing acute heat stress. Attempts to apply the inulin method to our needs were fruitless because of the prolonged time period required for equilibrium. Walser's radiosulfate method (1, 2) had appeal since it appeared suitable for repeated determinations within a short period of time.

Walser has suggested that within 15 to 20 minutes following a single intravenous injection, radiosulfate reached an apparent equilibrium between the vascular and interstitial spaces (1,2). He showed that the calculated volume of distribution of radiosulfate increased with time (1, 2) and that this increase could not be accounted for by urinary excretion (1, 2), fecal loss (3), or penetration of the muscle chloride space (4). He proposed that the 18-minute volume of distribution of radiosulfate could be used as an index of functioning extracellular fluid volume (1, 2).

The present report, dealing with the radiosulfate method, confirms and extends many of Walser's observations. In addition, it points out the desirability of calculating a "zero time" rather than an 18-minute volume of distribution, and proposes a modification of existing ways of calculating the "zero time" space.

METHODS

A. *Subjects:* Radiosulfate spaces were determined on 33 healthy individuals: eleven (Nos. 1–11) young, muscular soldiers; nine (Nos. 12–20) young, male medical students and physicians; five (Nos. 21–25) ambulatory, elderly males; and eight (Nos. 26–33) ambulatory, elderly females. Four patients with abnormalities of water metabolism were studied: two (Nos. 34, 35) were dehydrated and two (Nos. 36, 37) had ascites without peripheral edema.

In five subjects the radiosulfate space was redetermined at various intervals, ranging from 1 to 30 days, during which they continued their usual daily activities. Each of the eleven soldiers had a second determination following a three-week period of paratroop training.

B. *Procedure:* All subjects were studied in the morning after a twelve-hour fast. They were allowed water *ad libitum* during the preceding day and were given 200 ml. of water every two hours during the course of the test.

Approximately 100 microcuries of S^{35} as carrier-free sodium sulfate [3] were injected intravenously, from a calibrated syringe, over a period of one minute. An aliquot of the injected material was set aside as a standard. Timed blood samples were obtained from the arm opposite the site of the injection. Timed urine samples were obtained by indwelling catheters from subjects 21 through 35, and as voided samples in all others. Where catheters were used the bladder was washed with physiologic saline, followed by injection of air and manual expression. Ascitic fluid samples were obtained from indwelling polyethylene catheters with multiple openings.

In three subjects inulin space was simultaneously determined by the 5-hour constant infusion technique (4, 5). Inulin was determined in plasma and urine by Kendrick's modification (6) of the method of Harrison (7). The inulin space was calculated by dividing the inulin excreted in the urine (collected for 24 hours after completion of the infusion) by the plasma concentration at the end of the infusion. A timed blank correction was made on the urine sample. The space, thus calculated, was corrected for a plasma water content of 93 per cent.

C. *Method for the determination of radiosulfate:* S^{35} emits a low energy beta particle and has a physical half-life of 87.1 days. The chief problem concerned with its determination is the phenomenon of self-absorption.

As plasma is added to a fixed amount of S^{35}, the observed radioactivity decreases as the amount (or weight) of plasma increases. There is a rapid decrease in observed activity as dry weight increases from 0.1 to 12 mgm. Increments of weight about 12 mgm. produce a second slower rate of decrease in observed radioactivity.

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[2] The opinions expressed in this paper are those of the authors and do not necessarily represent the official views of any government agency.

[3] Supplied on allocation from the Atomic Energy Commission by Dr. Donald Tabern, Abbott Laboratories, North Chicago, Illinois.

This second slower rate of decrease may be expressed by the equation:

$$S = \frac{\log \frac{C_2}{C_1}}{W_2 - W_1}, \qquad (1)$$

where:

S = Slope (the exponential rate of decrease in observed activity per mgm. increase in sample weight)

C_2 = Observed counts per minute at Weight 2

C_1 = Observed counts per minute at Weight 1

W = Weight in mgm.

Knowing the slope (S), a sample of known weight and activity can be corrected to an activity of any arbitrary reference weight. Thus, samples of different weights may be made comparable. We selected 50-mgm. sample weight as an arbitrary reference point. In this situation, the equation for self-absorption correction becomes:

$$\log C_{50} = \log C_0 - S\,(W_0 - 50), \qquad (2)$$

where:

C_{50} = activity at 50-mgm. sample weight in counts per minute

C_0 = activity at the observed weight in counts per minute

S = exponential rate of decrease in observed activity with increasing weight

W_0 = observed weight in mgm.

The slope (S) was determined by preparing a family of 19 curves covering a weight range of 30 to 80 mgm. and a range of activity from 300 to 40,000 counts per minute. For each curve, activity was held constant and weight varied by adding plasma without radioactivity to the planchet. The slopes of these curves were calculated and found to be quite constant, having a mean of -0.00633 (S.D. $\pm$ 0.00063). Substituting this value in equation (2), the correction equation becomes:

$$\log C_{50} = \log C_0 + .00633\,(W_0 - 50). \qquad (3)$$

Serum was separated by centrifuging at 3,000 rpm. Duplicate 500-lambda aliquots of serum were pipetted into clean, preweighed, disposable, nickel-plated planchets, dried under a 150-watt light bulb and weighed again. Counting in a windowless gas-flow counter went on until a minimum of 2,500 counts were recorded. Urine samples and the standard solution, after appropriate dilution, were treated in a similar fashion save that waste plasma (free of radioactivity) was added to bring them into the desired weight range. The results obtained according to equation (3) were corrected for physical decay. The mean difference between counts on duplicate samples was less than 3 per cent. (Care was taken to avoid uneven drying and particulate matter in the samples.)

CALCULATIONS

The mathematical principles for calculating the volumes of distribution of metabolized and nonmetabolized substances have been set forth (7–10). In essence, for substances that are excreted but not metabolized, the volume of distribution of the material, at any time following its administration is equal to the amount remaining in the body at that time divided by the plasma concentration at the same time.

$$VD_t = \frac{D - E_t}{P_t}, \qquad (4)$$

where:

VD = Volume of distribution in ml. at time t

D = administered dose

E_t = amount excreted by time t

P_t = plasma concentration in units per ml. at time t.

For materials that are not metabolized, or that do not enter other fluid compartments, the calculated volume remains constant. For substances that are metabolized or that penetrate other fluid spaces, equation (4) is inadequate. To compensate, equation (4) is calculated for "zero time," that theoretical moment when all the injected material is uniformly distributed in its assumed volume of distribution without being subject to excretion, metabolism or diffusion into other fluid compartments.

$$VD_0 = \frac{D}{P_0}, \qquad (5)$$

where:

VD_0 = Volume of distribution, in ml., at "zero time"

D = administered dose

P_0 = extrapolated "zero time" plasma concentration in units per ml.

Equation (5) assumes that any loss of material from the measured space occurs at a uniform rate. No material can instantaneously mix in the plasma and equilibrate between intra- and extravascular fluid. Since plasma concentration is initially higher than that of extravascular fluid, the rate of initial loss in the urine is greater than that prevailing when plasma and extravascular fluid have equilibrated. The material excreted during the equilibrium period never contributes to the post-equilibrium plasma concentrations or their "zero time" extrapolation. Failure to correct for this excessive equilibrium time excretion results in an overestimation of the volume of distribution when calculated by equation (5). The degree of overestimation is proportional to the equilibrium time excretion. This error may be minimized by one of two calculations. First, one may calculate the volume of distribution at various post-equilibrium times, using equation (4), and extrapolate the volumes of distribution to "zero time." Secondly, one may plot the amount of material remaining in the body (dose minus cumulative excretion) against time and extrapolate to "zero time." This may be regarded as the "effective" dose, that part of the administered dose that eventually becomes distributed in the given volume of distribution. This "effective" dose divided by the "zero time" plasma concentration yields a "zero time" volume of distribution corrected for

the excessive urinary loss before equilibrium has been reached.

$$S_0 = \frac{R_0}{P_0}, \qquad (6)$$

where:

S_0 = "zero time" volume of distribution in ml.

R_0 = "effective dose" ("zero time" extrapolation of the amount of material remaining in the body)

P_0 = "zero time" plasma concentration in units per ml. obtained by extrapolation.

The "zero time" volumes of distribution presented in this report were calculated by equation (6). These were then corrected for a plasma water content of 93 per cent and the Gibbs-Donnan phenomenon, assuming the factor to be 0.90. Since plasma volume was not determined, the Gibbs-Donnan factor was applied to the entire volume of distribution rather than the interstitial fluid volume. No correction was made for urinary lag time since urine flow rates were generally good.

RESULTS

A. *The disappearance of radiosulfur from the plasma*

Figure 1 shows, by means of an example, the disappearance of S^{35} from the plasma following a single intravenous injection of sodium radiosulfate. Two rates of disappearance are evident. The first is rapid but of brief duration. Within 15 to 20 minutes it is replaced by a second slower rate that persists for at least 8 to 9 hours. The second slower rate was calculated for each of our subjects. The results are presented in Table I. These rates are such that the plasma radioactivity decreases 50 per cent in 2 to 4 hours.

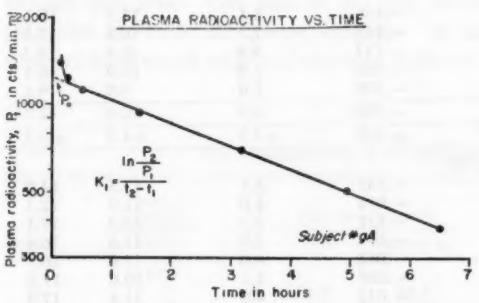

FIG. 1. THE DISAPPEARANCE OF RADIOSULFUR FROM THE PLASMA FOLLOWING A SINGLE INTRAVENOUS INJECTION

P_0 is the "zero time" extrapolation of the slower rate of disappearance.

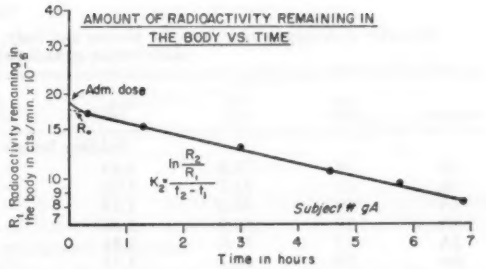

FIG. 2. THE AMOUNT OF RADIOSULFUR REMAINING IN THE BODY (DOSE-URINARY EXCRETION) FOLLOWING A SINGLE INTRAVENOUS INJECTION

R_0 is the "zero time" extrapolation of the slower rate of disappearance ("effective administered dose"). See text.

B. *The disappearance of radiosulfur from the body*

Intravenously injected S^{35} given as sodium sulfate is eliminated from the body chiefly by urinary excretion. Ninety-five per cent was recovered in the urine of Subject 14 within 5 days after administration. We have been able to account for only trace amounts in the sweat during a period of active sweating. Walser, Reid, and Seldin have found that fecal loss is negligible (3). Hence, for all practical purposes the amount of radioactivity remaining in the body may be expressed as the injected dose minus the urinary excretion.

Figure 2 shows the amount of S^{35} remaining in the body following a single intravenous injection. Two rates of disappearance are seen. The first is rapid and brief. It is replaced in 15 to 20 minutes by a second slower rate that lasts 8 to 9 hours.

The initial rapid rate of disappearance of S^{35} from the body is such that, on the average, 7.2 (S.D. $\pm$ 3.6) per cent of the administered dose is lost. This ranged from 1.0 to 19 per cent of the administered dose. Table I shows the data.

The second slow rate of disappearance of S^{35} from the body was calculated for each of our subjects (see Table I). This rate was such that 50 per cent of the injected dose was lost from the body in 4 to 8 hours. This was notably different from the rate of disappearance from the plasma (see above).

Renal clearance during the second slow phase of disappearance from the body was calculated for each subject. The mean clearance in ml. per

TABLE I

The rates of disappearance from the plasma and body, pre-equilibrium urinary excretion and zero time volume of distribution of radiosulfate in healthy individuals

Subject	Age *years*	Wt. *Kg.*	S.A. M^2	K_1* $\Delta ln/hr.$	K_2† $\Delta ln/hr.$	E. 3 hr.‡ *% adm. dose*	Vol. dist. *liters*	Vol. dist. *% body wt.*
Soldiers before paratroop training								
1A	22	73.5	1.89	−.170	−.108	2.3	16.4	22.3
2A	21	72.7	1.92	−.148	−.095	5.9	13.1	18.0
3A	19	63.6	1.78	−.139	−.100	5.1	11.9	18.8
4A	17	66.9	1.84	−.089	−.066	7.4	13.9	20.8
5A	17	71.0	1.84	−.145	−.131	5.1	14.4	20.3
6A	18	62.7	1.73	−.228	−.140	7.8	10.6	16.9
7A	18	74.7	1.96	−.174	−.116	7.5	14.7	19.7
8A	17	71.6	1.86	−.161	−.129	5.6	13.5	18.8
9A	19	74.6	1.92	−.181	−.110	4.2	12.2	16.3
10A	18	69.5	1.86	−.147	−.160	13.5	13.6	19.6
11A	19	70.9	1.85	−.140	−.132	9.5	13.6	19.0
Mean	18.6	70.2	1.86	−.157	−.117	6.7	13.4	19.1
Std. Dev.	±1.6	±4.1	±.07	±.034	±.040	±3.0	±1.5	±1.7
Soldiers after paratroop training								
1B	22	71.5	1.88	−.221	−.112	13.6	13.8	19.3
2B	21	71.4	1.90	−.175	−.179	11.1	14.2	19.9
3B	19	63.1	1.77	−.177	−.167	6.7	10.7	17.0
4B	17	66.6	1.84	−.206	−.251	7.7	10.8	16.2
5B	17	70.0	1.84	−.187	−.181	12.1	13.7	19.6
6B	18	64.2	1.74	−.222	−.084	3.4	10.3	20.2
7B	18	74.0	1.96	−.169	−.120	12.5	13.1	17.7
8B	17	69.7	1.84	−.209	−.186	10.6	12.1	17.1
9B	19	74.0	1.92	−.185	−.118	16.2	12.8	17.2
10B	18	68.4	1.85	−.169	−.096	6.6	14.3	20.8
11B	19	69.7	1.84	−.160	−.080	4.0	13.4	19.2
Mean	18.6	69.3	1.85	−.189	−.143	9.5	12.7	18.6
Std. Dev.	±1.6	±3.6	±0.06	±.022	±.054	±4.1	±1.5	±1.6
Young sedentary males								
12	31	82.5	1.97	−.167	−.131	15.3	11.6	14.1
13	28	61.0	1.74	−.183	−.189	5.8	9.6	15.8
14	30	78.5	1.99	−.174	−.139	6.6	13.0	16.5
15	23	70.6	1.86	−.151	−.111	12.8	15.5	21.9
16	22	67.1	1.75	−.209	−.158	19.2	10.6	15.8
17	23	79.6	1.96	−.168	−.175	13.0	13.5	17.0
18	30	67.7	1.87	−.141	−.213	2.5	11.4	16.8
19	25	78.8	2.01	−.166	−.144	10.1	13.5	17.2
20	25	58.6	1.69	−.222	−.183	4.0	9.0	15.4
Mean	26.3	71.6	±1.87	−.176	−.160	9.9	12.0	16.7
Std. Dev.	±3.5	±8.7	±0.12	±.026	±.032	±5.6	±2.1	±2.2
Elderly males								
21	63	77.7	1.83	−.168	−.106	4.5	12.5	16.1
22	61	62.3	1.71	−.132	−.125	7.2	10.8	17.3
23	61	70.9	1.76	−.150	−.142	8.6	10.8	15.2
24	63	60.9	1.73	−.171	−.065	1.0	10.0	16.4
25	65	95.5	2.14	−.191	−.100	1.0	9.0	9.4
Mean	62.6	73.5	1.83	−.162	−.108	4.5	10.6	14.9
Std. Dev.	±1.7	±14.1	±0.18	±.022	±.029	±3.5	±1.3	±3.2
Elderly females								
26	56	82.2	1.80	−.152	−.131	5.1	11.5	14.0
27	57	96.8	2.02	−.096	−.074	4.0	11.9	12.3
28	60	60.5	1.60	−.241	−.131	3.3	10.3	17.1
29	64	70.7	1.74	−.166	−.087	2.0	11.6	16.4
30	76	66.8	1.77	−.132	−.068	4.0	11.0	16.5
31	65	62.3	1.61	−.085	−.068	4.1	10.6	17.0
32	72	65.3	1.57	−.075	−.032	1.0	11.4	17.5
33	72	54.8	1.50	−.186	−.080	6.1	7.1	12.9
Mean	65.3	69.9	1.70	−.142	−.084	3.7	10.7	15.5
Std. Dev.	±7.8	±13.5	±0.17	±.018	±.011	±1.6	±1.5	±2.1

* K_1—Exponential rate of disappearance of radiosulfate from the plasma.
† K_2—Exponential rate of disappearance of radiosulfate from the body.
‡ Urinary excretion during the Equilibrium period expressed as E. 3 hr.-percentage of the administered dose.

min. per 1.73 m^2 was found to be 36.2 for 31 determinations on 22 young men, 25.8 for elderly men, and 21.7 for elderly women. The values for young men are comparable to those previously reported (2, 11, 12). The lower values in elderly people are probably related to the decrease in glomerular filtration rate that occurs with increasing age (13).

C. *The volume of distribution of radiosulfur as a function of time*

Figure 3 shows the volume of distribution of radiosulfate at various post-equilibrium times (calculated by equation *4*). The calculated volume increases exponentially with time. The rate of increase is the difference between the rate of disappearance of S^{35} from the plasma and from the body:

$$VD_t = S_0 e^{(k_2 - k_1)}, \qquad (7)$$

where:

VD_t = volume of distribution in ml., at time t

S_0 = "zero time" volume of distribution in ml. (obtained by extrapolation or equation *6*)

k_2 = exponential rate of disappearance of radiosulfur from the body

k_1 = exponential rate of disappearance of radiosulfur from the plasma

t = time.

Most of our subjects had volumes of distribution that increased with time. Six (No. 10A, 2B, 4B, 13, 17, 18), however, had volumes that decreased with time. Our data do not give an explanation for these decreasing volumes. A loss of water from the plasma or a return of radioactive sulfate to the plasma from some pool of high specific activity could result in such a phenomena.

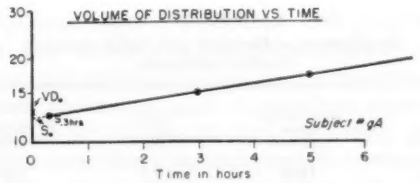

FIG. 3. THE CHANGE OF VOLUME OF DISTRIBUTION OF RADIOSULFUR, WITH TIME, FOLLOWING A SINGLE INTRAVENOUS INJECTION

S_0 is the extrapolated "zero time" volume, $S_{3\,hrs.}$ the equilibrium time volume and VD_0 the "zero time" volume of distribution calculated as the dose divided by the "zero time" plasma concentration.

D. *The zero time volume of distribution of radiosulfate in healthy individuals*

Data are presented in Table I. The mean "zero time" radiosulfate space was found to be 19.1 (S.D. ± 1.7) per cent of body weight in muscular young soldiers (1A to 11A) before 3 weeks of paratroop training and 18.7 (S.D. ± 1.6) per cent of body weight after training (1B to 11B). The mean "zero time" radiosulfate space was 16.7 (S.O. ± 2.2) per cent of body weight in sedentary young males, 14.9 (S.D. ± 3.2) per cent of body weight in elderly males, and 15.5 (S.D. ± 2.1) per cent of body weight in elderly females.

The difference between the values obtained before and after paratroop training was not significant. The muscular soldiers had significantly larger radiosulfate spaces than the sedentary young males ($P < .01$). The differences between sedentary young and elderly males and between elderly men and women were not significant.

TABLE II

The reproducibility of the zero time radiosulfate space in healthy individuals

Subject	Interval between determinations	First determination		Second determination		Difference	
		liters	*% body wt.*	*liters*	*% body wt.*	*liters*	*% body wt.*
	days						
12	1	11.6	14.1	12.7	15.4	+1.1	+1.3
13	7	9.6	15.8	9.2	15.8	−0.4	−0.0
15	1	15.5	21.9	15.1	21.4	−0.4	−0.5
19	1	13.5	17.2	13.3	17.0	−0.2	−0.2
26	30	12.2	14.8	11.5	14.0	−0.7	−0.8
Mean		12.48	16.76	12.36	16.72	−0.12	−0.04

TABLE III

Simultaneous radiosulfate and inulin spaces in healthy individuals

Subject	Radiosulfate space in liters	Inulin space in liters	Ratio: Radiosulfate / Inulin
22	10.8	10.5	1.03
23	10.8	10.9	0.99
28	10.3	11.1	0.93

E. *The reproducibility of the zero time radiosulfate space*

Five individuals (Nos. 12, 13, 15, 19, 26) had second determinations at various intervals ranging from 1 to 30 days following their initial test. Data are presented in Table II. The mean difference between the two determinations was − 0.12 liter with a range of + 1.1 to − 0.7 liters. The mean difference expressed as percentage of body weight was − 0.04 with a range of + 1.3 to − 0.8.

F. *Comparison of the zero time radiosulfate space with inulin space*

Table III shows the values obtained when we simultaneously determined "zero time" radiosulfate and 5-hour constant-infusion inulin spaces on three subjects. The mean radiosulfate-inulin space ratio was 0.98.

G. *The volume of distribution of radiosulfate in patients with abnormal water metabolism*

Table IV presents data on 4 patients. Subject 34 was a 60-year old female admitted in diabetic acidosis. Clinically, she was moderately dehydrated. Her "zero time" radiosulfate space was 8.2 liters or 10.5 per cent of body weight. Subject 35 was a 65-year old man who had suffered a cerebro-vascular accident approximately 48 hours before being found and brought to the hospital. He was severely dehydrated, oliguric and had hyperchloremia and hypernatremia. His radiosulfate space was 4.9 liters or 7.2 per cent of body weight.

Subject 36 was a 42-year old man with rheumatic mitral and tricuspid stenosis and insufficiency. He had 7,600 ml. of ascitic fluid obtained by paracentesis, but no peripheral edema. His radiosulfate space [4] was 16.3 liters or 26.2 per cent of body weight. Subtracting his ascitic fluid volume from his radiosulfate space gives a value of 8.7 liters or 16.8 per cent of his weight after paracentesis, a value well within the normal range. Subject 37 was a 60-year old man with Laennec's cirrhosis and ascites (3,600 ml.) but no peripheral edema. His radiosulfate space was 16.7 liters or 22.8 per cent of his weight. After subtraction of

TABLE IV

The zero time radiosulfate space in abnormal individuals

Subject	Clinical diagnosis	Abnormality of water metabolism	V.D. *liters*	V.D. *% body wt.*
34	Diabetes mellitus in acidosis	Moderate dehydration	8.2	10.5
35	C.V.A. with unconsciousness	Severe dehydration	4.9	7.2
36	Rheumatic heart disease	Ascites (7,600 ml.) without peripheral edema	16.3	26.2
37	Laennec's cirrhosis	Ascites (3,200 ml.) without peripheral edema	16.7	22.8

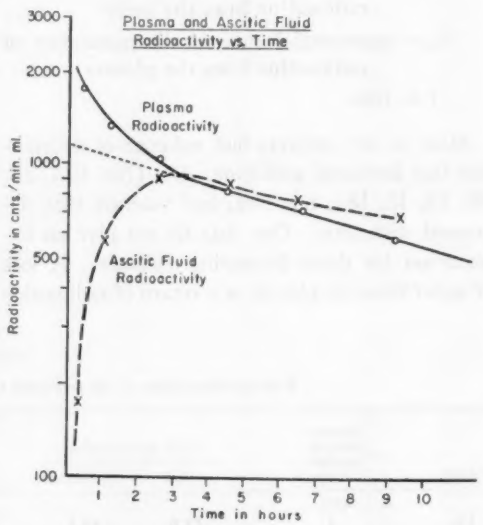

FIG. 4. THE CHANGE IN PLASMA AND ASCITIC FLUID RADIOACTIVITY, WITH TIME, FOLLOWING A SINGLE INTRAVENOUS INJECTION OF RADIOSULFUR

[4] For the subjects with ascites the volume of distribution was calculated at the time of equilibrium between plasma and ascitic fluid using the calculation described by Deane (14).

his ascitic fluid volume from his radiosulfate space he had a value of 13.1 liters or 18.7 per cent of his "dry" weight.

Figure 4 shows plasma and ascitic fluid radioactivity following a single intravenous injection of S^{35} in Subject 37. A transient equilibrium between plasma and ascitic fluid concentrations occurred 3.4 hours following injection. In Subject 36, who had a larger amount of ascitic fluid, equilibrium occurred at 5.1 hours. These equilibrium times are comparable to those reported by Madison, Teng, Seldin, Reid, and MacDonald (15).

DISCUSSION

A. *The zero time radiosulfate space*

In theory, the extracellular fluids consist of plasma, lymph, interstitial fluid, cerebrospinal fluid, aqueous humor, vitreous humor, synovial, pleural and pericardial fluids, glandular secretions and urine. These anatomic entities, however, do not behave as a single physiologic unit. The physiologic extracellular water consists of the plasma and those fluid compartments with which it freely exchanges ions and small molecules. It differs from the anatomical extracellular fluid by excluding secretions and possibly the ocular humors and cerebrospinal fluid. The relationship of ground substance and its bound water to either the anatomic or physiologic extracellular water is not clear. Neither the anatomic nor the physiologic extracellular fluid can be measured by direct means. They must be estimated from the dilution of an administered chemical. As yet, no chemical has been found which is confined to the physiologic extracellular water. However, since ions and small molecules generally diffuse rapidly through the physiologic extracellular water and slowly into other fluid compartments an estimation of extracellular water can be made. The problems dealing with estimations of extracellular fluid volume, *in toto*, and in various tissues have recently been reviewed and discussed by Manery (16).

The relationship of the radiosulfate space to an anatomically defined extracellular space is obscure. There are not sufficient data to define accurately the distribution of radiosulfate. Very little radiosulfate has been found in gastric juice (2) or sweat. Sulfate does not readily penetrate brain tissue or cerebrospinal fluid from the blood (17). It has been found to equilibrate with peritoneal fluid and therefore it should equilibrate with pleural and pericardial fluid. It has been shown that the radiosulfate space of muscle is approximately 20 per cent smaller than the muscle chloride space and that it remains constant between 20 and 250 minutes following its administration (4). This is probably due to the higher concentration of chloride in muscle connective tissue and the slowness or lack of penetration of sulfate into connective tissue water. Data are lacking on the distribution of radiosulfate in lymph, interstitial fluid, synovial fluid, and the ocular humors. Radiosulfate does equilibrate with edema fluid water in about 11 hours (15). From the available data it appears that radiosulfate space is not a measure of an anatomical extracellular space, but is an index of physiologic extracellular water, *i.e.*, the plasma and those fluid compartments with which it rapidly exchanges ions and small molecules.

From the data of Sheatz and Wilde on the transcapillary migration rate of radiosulfate (17), and the initial rapid rate of disappearance of radiosulfate from the plasma noted by Walser, Seldin, and Grollman (1, 2), it appears that radiosulfate has a rapid diffusion into an extravascular water compartment that we presume is the physiologic extracellular water. Since radiosulfate is not bound to plasma proteins,[5] it seems reasonable to assume that the concentration of radiosulfate in plasma is representative of that in the physiologic extracellular space when the Gibbs-Donnan phenomenon is taken into account. However, since the calculated radiosulfate space increases with time, it is apparent that the isotope is not confined to the physiologic space. It must be metabolized or penetrate other body fluid compartments. The extent and rate of loss of radiosulfate, in this fashion, will be discussed below. Suffice it to say here, that the loss occurs at a relatively slow rate and that the error introduced by such a loss can be minimized by calculation of a "zero time" space.

Whether one calculates a "zero time" or an 18-minute volume of distribution, as Walser did, it cannot be assumed, as has been proposed (2), that four per cent of the injected material is excreted in the urine during the equilibrium period. Our

[5] Plasma radiosulfur can be completely dialyzed and is unprecipitable with trichloracetic acid and phosphotungstic acid.

data show that the urinary excretion during the assumed equilibrium time of 18 minutes is quite variable and ranges from 1 to 19 per cent of the injected material.

The relationship of the radiosulfate space to other indices of extracellular fluid volume is not precisely established. The "zero time" volumes of distribution of radiosulfate, seen in our healthy individuals, are comparable to those previously reported (1, 2, 18), and are generally smaller than the volumes of distribution of chloride (19, 20), bromide (20), and thiocyanate (19, 21). The discrepancies might be due to the penetration of the latter substances into connective tissue water (22), into cell water (23–25), sequestration in the gastrointestinal tract (26), or, in the case of thiocyanate, binding to protein (27). Recently, Berson and Yalow (28) have shown that calculation of the volume of distribution of radiosodium and radiobromide from their early rates of disappearance from the plasma yields values that amount to approximately 18 per cent of body weight, even though their ultimate volumes of distribution are much greater. These values are comparable to those found for radiosulfate and suggest that the discrepancy between chloride and radiosulfate spaces might be eliminated by calculation of chloride space from its early rate of disappearance from plasma.

The values we have obtained for "zero time" radiosulfate space are comparable to those reported for the 5-hour constant-infusion inulin space (5, 29, 30), constant-infusion sucrose space (14, 19, 31), and "zero time" thiosulfate space (32). They are slightly smaller than reported mannitol spaces (33).[6] Walser found a ratio of 0.95 between simultaneously determined radiosulfate and inulin spaces. On three subjects we also found good correspondence between inulin and radiosulfate spaces. This apparent agreement of these several methods may be coincidental. Becker and Heinemann (18) found that the inulin-radiosulfate ratio approached unity, but that the coefficient of correlation between the two was only 0.3. Swan, Madisso, and Pitts (34) recently determined simultaneous "zero time" mannitol and inulin, sucrose, raffinose, thiosulfate and radiosulfate spaces in nephrectomized dogs. Using mannitol as a reference they found ratios of 0.74 for inulin, 0.87 for raffinose, 0.91 for sucrose, 0.99 for thiosulfate and 1.0 for radiosulfate. This may be due to a relatively slow penetration of the larger inulin, raffinose and sucrose molecules into some relatively inaccessible fraction of extracellular water. The data of Cotlove (35) and Nichols, Nichols, Weil, and Wallace (36) suggest that this may be some connective tissue phase.

[6] These mannitol spaces were not corrected for the excessive urinary loss of mannitol during the equilibrium period and therefore are too large.

B. *The slowly exchanging sulfate pool*

Since injected S^{35} leaves the plasma at a faster rate then it appears in the urine it is apparent that it is being metabolized or entering fluid compartments other than its "zero time" volume of distribution. We have attempted to shed some light on this slowly exchanging pool using the mathematical approach presented in the Appendix. This approach has been applied to the data obtained on the eleven young soldiers and the results are given in Table V and illustrated in Figure 5.

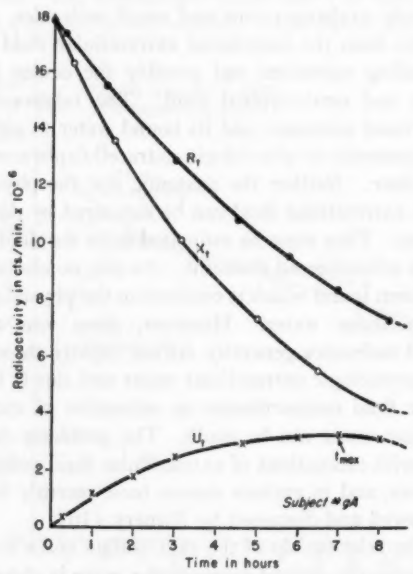

FIG. 5. THE EXTRA-URINARY LOSS OF RADIOSULFUR (U_t) FOLLOWING A SINGLE INTRAVENOUS INJECTION

R_t represents the disappearance of radiosulfur from the body; X_t the disappearance of radiosulfur from the physiologic extracellular space and t_{max} the time at which the extra-urinary loss of radiosulfur is maximum.

TABLE V

The extra-urinary loss of radiosulfate in eleven soldiers before and after paratroop training

Subject	Before				After			
	t_{max}*	Ut_{max}†	VU‡ *liters*	VU‡ *% body wt.*	t_{max}	Ut_{max}	VU *liters*	VU *% body wt.*
1	7.3	16.0	9.0	12.2	6.3	22.9	13.6	19.0
2	8.3	14.5	8.5	11.7	—	—	—	—
3	8.5	11.9	4.6	7.2	5.8	2.2	0.7	1.0
4	12.7	10.4	4.7	7.0	—	—	—	—
5	7.3	2.7	0.8	1.1	5.5	1.1	0.3	0.4
6	5.6	17.1	7.2	11.5	7.1	33.4	22.0	34.3
7	7.0	14.3	7.6	10.2	7.0	11.8	5.5	7.5
8	6.9	8.5	3.5	4.9	5.1	4.1	1.5	2.2
9	7.0	18.0	7.9	10.6	6.7	14.2	7.2	9.8
10	—	—	—	—	7.8	19.7	10.9	15.9
11	7.4	2.2	0.9	1.3	8.7	24.6	13.4	19.3
Mean	7.8	11.6	5.5	7.8	6.7	14.9	8.3	12.6
Std. Dev.	±1.90	±5.63	±3.04	±4.20	±1.14	±11.19	±7.29	±11.13

* t_{max} = The time of maximum extra-urinary loss of radiosulfate in hours.
† Ut_{max} = The maximum extra-urinary loss of radiosulfate expressed as per cent of the administered dose.
‡ VU = The volume that the extra-urinary loss would occupy if it were contained in a fluid compartment in simple diffusion equilibrium with the "extra-cellular space" (see text).

The maximum amount of radiosulfate entering the slowly exchanging pool was found to be approximately 10 to 15 per cent of the injected dose. This maximum was reached 6 to 8 hours after injection. Considerable variation was noted. The maximum amount of radiosulfate entering the slowly exchanging pool during the first twenty minutes after injection was 1.3 (S.D. ± 1.2) per cent of the injected dose. It must be stressed that this is an over-estimation since during the first twenty minutes after the injection the concentration of radiosulfate is greater in the plasma than in the interstitial fluid.

With the thought that the slowly exchanging pool might represent a fluid compartment, we attempted to calculate its volume using the principles set forth by Best (37) in his work with fluid analogues. The calculated volumes varied from 0.3 to 22.0 liters (see Table V). They were not reproducible. They do not conform to any known fluid compartment. We can only conclude that the extra-urinary loss of radiosulfur is not due to simple diffusion from "physiologic extracellular water" to some other fluid compartment.

We do not know what the slowly exchanging sulfate pool represents. It may be the net result of several simultaneously occurring phenomena. Everett and Simmons (38) report that in the rat, S^{35} is excreted in the bile and upper gastrointestinal tract and subsequently is reabsorbed in the large gut. It is incorporated into taurocholic acid (39). Dziewiatkowski, Benesch, and Benesch (40, 41) report that S^{35} is concentrated in cartilage (as chondroitin sulfate) and bone marrow. Radiosulfate enters into the formation of ethereal sulfate (42, 43). Ling feels that some sulfate enters muscle cells (44).

SUMMARY AND CONCLUSIONS

1. A method for determining radiosulfate in plasma and urine has been presented.

2. The disappearance of radiosulfate from the plasma and the body was discussed. A method of calculating a "zero time" radiosulfate space corrected for excessive equilibrium time excretion was derived. This calculated space has some advantages over the equilibrium time space and the "zero time" space uncorrected for urinary excretion.

3. Corrected "zero time" radiosulfate spaces were determined on 11 soldiers, 9 sedentary young males, 5 elderly males, and 8 elderly females. They were found to average 19.1, 16.7, 14.9, and 15.5 per cent of body weight, respectively. This "zero time" space was found to be reproducible in the steady state and to vary in the clinically indicated degree and direction in patients with abnormal water metabolism. Approximately 5 hours were required for radiosulfur to equilibrate between plasma and ascitic fluid.

4. The distribution of radiosulfate and the relationship of radiosulfate space to other indices of extracellular fluid volume have been discussed. It is concluded that the "zero time" radiosulfate space is an index of physiologic extracellular water.

5. A method of calculating the slowly exchanging sulfate pool has been presented. The significance of this pool has been discussed.

APPENDIX

Injected S^{35} leaves the plasma at a faster rate than it appears in the urine. Assuming that the "zero time" radiosulfate space remains constant, the extra-urinary loss of S^{35} during the post-equilibrium period can be calculated. This is the amount remaining in the body (dose minus excretion) minus that remaining in the "zero time" space. The amount remaining in the "zero time" space is the product of its volume and its concentration:

$$X_t = cS_0P_0e^{K_1t}, \qquad (8)$$

where:

X_t = radioactivity remaining in the "zero time" radiosulfate space at time t in counts per minute

c = constant correcting for plasma water content and Gibbs-Donnan effect (assumed to be 1.194)

S_0 = "zero time" radiosulfate space in ml.

P_0 = "zero time" plasma concentration in counts per minute per ml.

K_1 = exponential rate of disappearance of radiosulfate from the plasma

t = time in hours.

The S^{35} remaining in the body after the equilibrium period is:

$$R_t = R_0e^{K_2t}, \qquad (9)$$

where:

R_t = amount of radiosulfate remaining in the body at time t in counts per minute

R_0 = amount of radiosulfate remaining in the body at "zero time" (obtained by extrapolation) in counts per minute

K_2 = exponential rate of disappearance of radiosulfate from the body

t = time in hours.

The amount of S^{35} being metabolized or entering fluid compartments other than its original volume of distribution is the difference between equations (*9*) and (*8*). This simplifies to:

$$U_t = R_0(e^{K_2t} - e^{K_1t}), \qquad (10)$$

where:

U_t = the amount of radiosulfate, in counts per minute, lost by extra-urinary sources by time t.

It is apparent that the extra-urinary loss of S^{35} reaches a maximum and thereafter decreases (see Figure 5). The time at which this maximum is reached can be calculated from equation (*10*):

$$t_{max} = \frac{\ln \frac{k_2}{k_1}}{k_2 - k_1}, \qquad (11)$$

where:

t_{max} = time of maximum extra-urinary loss of radiosulfate in hours

k_1, k_2 = the exponential rates of loss of radiosulfate from the plasma and the body, respectively.

Knowing the time at which the extra-urinary loss is maximum the maximum amount may be calculated.

$$Ut_{max} = R_0e^{t_{max}}(k_2 - k_1). \qquad (12)$$

If one assumes that the extra-urinary loss of S^{35} is due to simple diffusion into a second fluid compartment, then it is reasonable to assume that there is no net exchange between the two compartments when the radioactivity in the second compartment reaches a maximum. At this time the concentrations in the two compartments would be equal and would be determinable since the concentration in one can be measured. Thus, knowing the total amount of S^{35} (by equation *12*) and the concentration of S^{35} in the second compartment, its volume can be calculated:

$$VU = \frac{Ut_{max}}{cPt_{max}}, \qquad (13)$$

where:

VU = the volume of the "second radiosulfate space" in ml.

ACKNOWLEDGMENT

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QUANTITATIVE URINARY COPROPORPHYRIN EXCRETION AND ITS RELATION TO EDATHAMIL CALCIUM DISODIUM ADMINISTRATION IN CHILDREN WITH ACUTE LEAD INTOXICATION [1, 2]

By J. JULIAN CHISOLM, Jr. and HAROLD E. HARRISON

(From the Department of Pediatrics, Johns Hopkins Hospital and School of Medicine and the Pediatric Division of the Baltimore City Hospitals, Baltimore, Md.)

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The disturbance of porphyrin synthesis induced by lead in both humans and experimental animals results in an increased excretion of porphyrins in the urine. The factors contributing to the porphyrinuria of lead poisoning remain incompletely understood. For the available evidence on the topic the reader is referred to several excellent reviews (1–4). Several groups of investigators (5–7), have reported data indicating a correlation between the concentration of coproporphyrin in urine and the concentrations of lead in the blood and urine of untreated industrial lead workers. These investigators used semiquantitative techniques for the estimation of coproporphyrin in urine. Bashour (8) has also reported a positive correlation ($r = 0.37$) between blood lead concentration and the quantitative excretion of coproporphyrin in the urine of lead-exposed adults.

During a study of acute and chronic lead poisoning in children certain quantitative relationships became apparent between the total daily urinary coproporphyrin output and a) the urine lead output induced by edathamil calcium disodium administration,[3] and b) whole blood lead concentration.

MATERIALS AND METHODS

The data to be presented are based upon serial determinations of 24-hour urinary coproporphyrin output, 24-hour urine lead output and whole blood lead concentrations in children during and following their recovery from acute lead encephalopathy. All of these children were treated initially with one or two courses of edathamil calcium disodium, which chelates with lead and increases its excretion in the urine. The dosage used in all patients was 75 mg. per Kg. per day, and this was given in divided doses subcutaneously or intramuscularly for periods of five to seven days with an interval of 14 to 21 days between courses. The criteria, upon which the diagnosis of acute lead encephalopathy was based, are reported in another paper (9). The subjects ranged in age from 18 months to 4½ years. Following recovery from their initial episode of encephalopathy none of the subjects included in the present report had any known reexposure to abnormal sources of lead.

Urinary coproporphyrin [4] output was determined by the photofluorimetric technique of Schwartz, Zieve, and Watson (10). In this laboratory determinations of total urinary coproporphyrin output in control subjects gave values which agreed with the normal range for children reported by Hsia and Page (11) and Nevé and Aldrich (12) (0 to 75 γ per 24 hrs. for children weighing less than 80 lbs.). The age and weight distributions of the control subjects were comparable to those of the patients in the present study.

For the determination of lead in urine specimens were wet-digested and their lead content determined colorimetrically with dithizone according to the method of Bessman and Layne (13).

Twenty-four hour urine collections were carried out on a metabolism frame (14) with precautions for the separation of urine and feces. Lead-free collection apparatus was used throughout. When specimens for coproporphyrin analysis were collected, 1 to 3 grams of sodium carbonate, reagent grade, were added to the collection bottle as preservative. Five-gram samples of the lot of sodium carbonate used contained no detectable quantity of lead. Daily urinary creatinine excretion was determined as a further check on the completeness of each urine collection.

Whole blood lead concentration was determined by the Division of Laboratories of the Baltimore City Health Department by a wet-digestion dithizone technique (15).

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[2] Read in part before the Combined Meeting of the American, British and Canadian Pediatric Societies, Quebec City, Quebec, June 1955.

[3] Edathamil calcium disodium used during this study was kindly supplied by Riker Laboratories, Inc., Los Angeles, California.

[4] The authors wish to thank Dr. Samuel Schwartz for his generous gift of coproporphyrin which was used as a reference standard.

Specimens of blood were collected with the lead-free apparatus supplied by that laboratory.

RESULTS

In children with lead intoxication a positive correlation is found between the 24-hour urinary coproporphyrin output just prior to the parenteral administration of edathamil calcium disodium and the lead output during the first 24-hour period following institution of treatment with this chelating agent. The relationship is an exponential one and is shown graphically in Figure 1. In this diagram the urinary coproporphyrin output is plotted logarithmically on the ordinate against the arithmetic plot of urine lead output on the abscissa so that a straight line is obtained. The slope of this regression (b) is 0.1275; its correlation coefficient ($r = 0.8213$) is statistically significant ($P < 0.03$) when $N = 7$. Since treatment with edathamil calcium disodium could not be deferred initially in patients with acute lead encephalopathy in order to obtain pre-treatment urinary coproporphyrin values, the data shown in Figure 1 were obtained from patients during the recovery phase. Each point on the curve was obtained from a different patient. Two of the patients had had acute lead encephalopathy one year previously and were clinically asymptomatic. In the remaining five the determinations were made two to three weeks after hospital admission and the initial

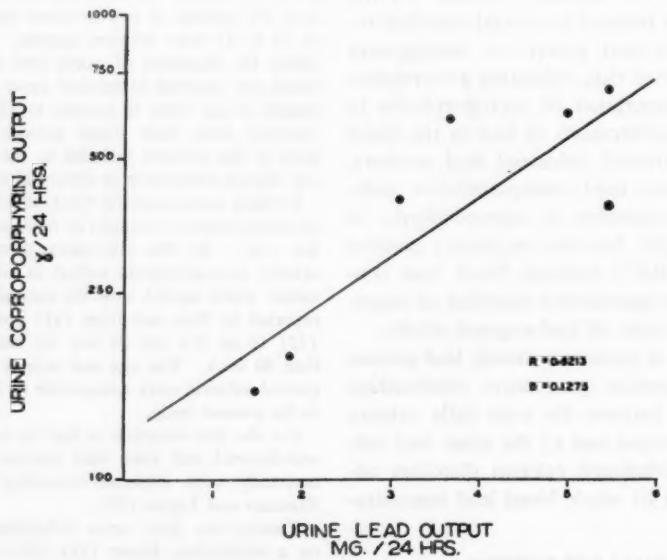

FIG. 1. RELATION BETWEEN PRE-TREATMENT URINE COPROPORPHYRIN OUTPUT AND URINE LEAD OUTPUT DURING FIRST 24 HOURS OF EDATHAMIL CALCIUM DISODIUM ADMINISTRATION

In this graph the urine lead (mg. per 24 hrs.) is the quantity excreted during the first 24 hours following parenteral administration of edathamil calcium disodium; whereas, the urine coproporphyrin (γ per 24 hrs.) is that determined just prior to institution of therapy. Urine lead output is plotted arithmetically on the abscissa and urine coproporphyrin output on the ordinate on a logarithmic scale. The relationship can be expressed mathematically by the following equation:

$$\log y = \log a + b\,x.$$

In this equation y = urine coproporphyrin output (γ per 24 hrs.); a = intercept of y on its axis; b = regression coefficient; and x = urine lead output (mg. per 24 hrs.). For this regression $b = 0.1275$ and the correlation coefficient $r = 0.8213$ for which $P < 0.03$ but > 0.02 when $N-2 = 5$.

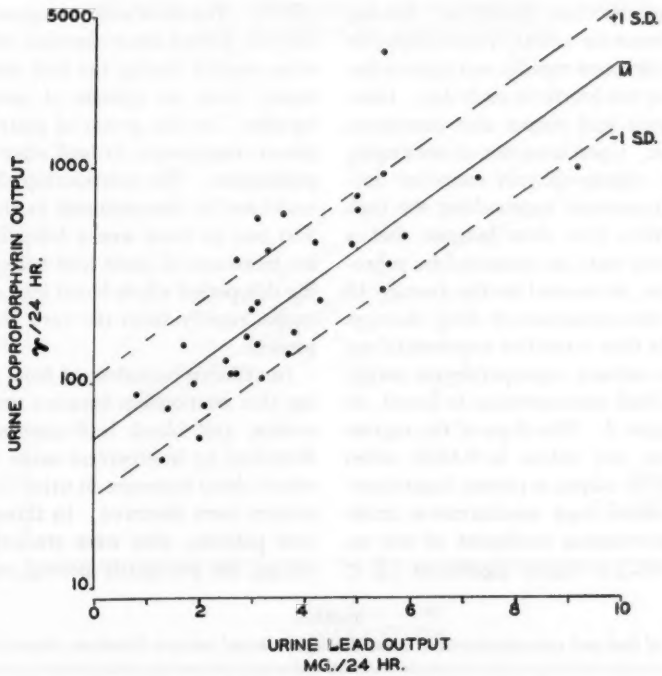

FIG. 2. RELATION BETWEEN SIMULTANEOUS URINARY EXCRETION OF LEAD AND COPROPORPHYRIN DURING EDATHAMIL CALCIUM DISODIUM ADMINISTRATION

In this figure the urine lead output (mg. per 24 hrs.) during edathamil calcium disodium treatment is plotted on the abscissa against urinary coproporphyrin output (γ per 24 hrs.) on the ordinate on a logarithmic scale. Each point represents a single day's excretion. The mathematical relationship is the same as that indicated for the relationship depicted in Figure 1. For the relationship shown above, the mean regression line is drawn as a solid line and ± 1 S.D. of y on x as interrupted lines.

For this regression: $b = 0.1762$
$r = 0.7642$ $(P < 0.001)$
$N = 33$

treatment of acute lead encephalopathy. The coproporphyrin determinations were made on 24-hour urine samples obtained one to three days prior to the institution of a course of edathamil calcium disodium treatment. This was done in order that any possible effect of the drug upon urinary porphyrin excretion independent of its effect upon lead metabolism might be eliminated.

A similar correlation is also found between the simultaneous urinary excretion of coproporphyrin and of lead during the parenteral administration of edathamil calcium disodium. This is shown in Figure 2. This correlation is demonstrable both during the acute and convalescent phases of lead encephalopathy if patients with marked oliguria during the acute illness are excluded. The relationship is again an exponential one. The data plotted in Figure 2 were computed from 33 simultaneous determinations of lead and coproporphyrin in urine during 14 courses of therapy in nine patients. The slope of this regression (b) is 0.1762; the correlation coefficient ($r = 0.7642$) is statistically highly significant ($P < 0.001$).

In Table I are shown the changes in the excretion of coproporphyrin and lead in the urine of children with lead poisoning during the adminis-

tration of edathamil calcium disodium. During the period of treatment the urinary coproporphyrin excretion usually decreases rapidly and approaches the normal range by the fourth to sixth day. Likewise, the daily urine lead output also decreases; but at a lesser rate. Upon cessation of treatment, however, urinary coproporphyrin excretion frequently returns to amounts approaching the high pre-treatment levels. Our data indicate that a new relatively steady state, as measured by coproporphyrin excretion, is reached on the average 14 to 21 days after discontinuance of drug therapy.

At and after this time a positive exponential relationship between urinary coproporphyrin output and whole blood lead concentration is found, as is indicated in Figure 3. The slope of the regression between these two values is 0.1850, when urine coproporphyrin output is plotted logarithmically and whole blood lead concentration arithmetically. The correlation coefficient of the regression ($r = 0.6642$) is highly significant ($P < 0.001$). The data were computed from 70 simultaneous, paired determinations in 15 patients who were studied during the first year after their recovery from an episode of acute lead encephalopathy. In this group of patients there was no known reexposure to lead after their initial hospitalization. The relationship shown in Figure 3 could not be demonstrated in children during the first two to three weeks following hospitalization for treatment of acute lead encephalopathy. During this period whole blood lead concentrations decrease rapidly from the very high levels initially present.

In children convalescent from acute lead poisoning this relationship between urine porphyrin excretion and blood lead concentration was also disturbed by intercurrent acute infections, during which sharp increases in urine coproporphyrin excretion were observed. In three of five convalescent patients, who were studied during such illnesses, the previously normal or slightly elevated

TABLE I

Output of lead and coproporphyrin in urine during edathamil calcium disodium administration *

Patient	Age mo.	Urine excretion	Treatment during acute lead encephalopathy: Initial course, Day of treatment period 1	2	3	4	5	6	7	Second course, Day of treatment period 1	2	3	4	5	6	Treatment during convalescence, Day of treatment period 1	2	3	4	5	6
A. W.	34	UCP†			1,022		920		277	650			106								
		Pb‡	12.1	11.4	9.2	9.5	7.3		5.5	5.0	5.2	3.2	3.2	4.5	4.7						
R. S.	18	UCP	450		138	129					45		89								
		Pb	4.9	5.1	3.7	2.5	1.9			1.5	1.3	1.1	0.80	0.62							
A. P.	25	UCP		452		222					239				75						
		Pb	4.1	4.2	4.1	3.0	1.7			2.7	3.1	4.1	2.0	1.5	1.4						
C. R.	26	UCP		416		494				364		248		166							
		Pb	4.0	3.3	6.0	5.9	6.4			5.6	3.2	4.3	3.4	2.9							
A. S.	22	UCP		746		379		150	150	594											
		Pb		5.0	3.8	2.7	2.4	3.1	1.7	3.1	2.1										
M. K.	30	UCP		3,430		173															
		Pb	6.2	5.5	4.1	3.8	2.5														
L. W.	23	UCP								615				113							
		Pb								3.6	3.4	2.7		2.6							
W. T.	54	UCP															950	113			
		Pb														5.2	5.5	2.7	1.4	0.39	
B. H.	36	UCP														100	80		56		
		Pb														1.9	2.1	2.2	2.0	2.4	1.1

† Urine coproporphyrin = γ/24 hrs.
‡ Urine lead = mg./24 hrs.
* This table presents the simultaneous daily urinary output of lead and coproporphyrin during parenteral edathamil calcium disodium administration. The ages shown are those at the time of onset of acute lead encephalopathy in each patient. "Initial Course" refers to treatment instituted at the time of onset of encephalopathy. "Second Course" refers to treatment begun 14–21 days after the "initial course." Patients B. H. and W. T. received treatment 10 months and 12 months, respectively, after onset of acute lead encephalopathy.

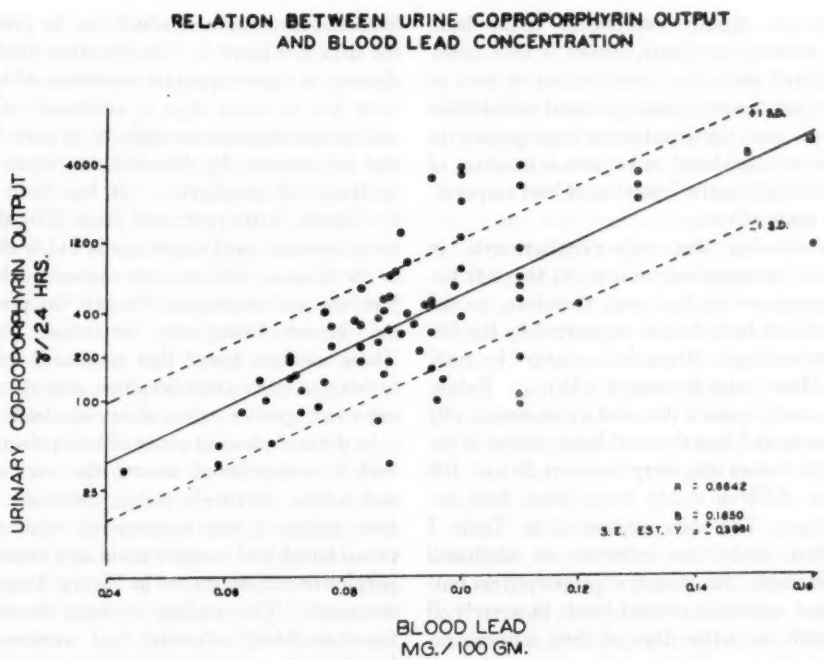

FIGURE 3

The data shown in the graph are based upon 70 simultaneous determinations of whole blood lead concentration and 24 hr. urinary coproporphyrin output in 15 patients made two weeks or longer after completion of edathamil calcium disodium administration. These data were obtained in patients in whom there was no known reexposure to lead following the initial illness. Whole blood lead concentration (mg. Pb per 100 gm. whole blood) is plotted arithmetically on the abscissa against the urinary coproporphyrin output (γ per 24 hrs.) which is plotted on the ordinate on a logarithmic scale. The mean regression line is shown as a solid line; while ± 1 S.D. of y on x from this mean regression is indicated by the broken lines.

For this regression: $b = 0.1850$
$r = 0.6642$ $(P < 0.001)$
$N = 70$

coproporphyrin outputs rose to levels of 500 to 1,000 γ per 24 hrs. during the infection. Their blood lead concentrations remained unchanged in the range of 0.05 to 0.075 mg. lead per 100 gm. of whole blood. In the absence of infection in convalescent lead-poisoned patients this range of whole blood lead concentration is not ordinarily associated with increases in urinary coproporphyrin output above 150 γ per 24 hrs.

DISCUSSION

The present studies in children with lead poisoning demonstrate a positive exponential correlation between daily total urinary coproporphyrin excretion and that quantity of lead in the body which is available for chelation and urinary excretion under the influence of edathamil calcium disodium (Figures 1 and 2). Foreman and Trujillo (16) have shown in humans that edathamil calcium disodium rapidly diffuses into all tissues with the exception of the erythrocytes and spinal fluid. The data of Rubin (17) indicate that in experimental animals this chelating agent removes lead primarily from non-skeletal tissues and enhances its excretion in urine and its storage in cancellous bone. It seems reasonable to assume, therefore, that the initial output of lead in the urine following edathamil calcium disodium administra-

tion represents rapidly mobilizable tissue lead. The total urinary porphyrin output is then probably correlated with the concentration of lead in the various soft tissues of lead-poisoned individuals. If this be the case, the quantitative coproporphyrin output can be considered to provide a measure of the "metabolically active" portion of lead responsible for its toxic effects.

The postulation that coproporphyrinuria in plumbism is quantitatively related to the soft tissue concentrations of lead and, therefore, to the toxic fraction of body lead is supported by the following observations. From data reported by Aub, Fairhall, Minot, and Reznikoff (18), by Kehoe, Thamann, and Cholak (19), and by ourselves (9) it can be estimated that the total lead content of the non-skeletal tissues may vary between 20 and 100 mg. Pb in children dying from acute lead encephalopathy. The data presented in Table I indicate that, under the influence of edathamil calcium disodium, the urinary coproporphyrin output does not approach normal levels in acutely ill patients until the latter days of drug administration, when the cumulative urinary excretion of lead reaches 20 to 50 mg. It was also observed clinically in the majority of acute cases of severe encephalopathy that subsidence of coma, convulsions and increased intracranial pressure usually occurred on the second or third day after institution of therapy. In individual patients the subsidence of these symptoms roughly coincided, during their initial course of treatment, with the time at which the urinary coproporphyrin excretion decreased.

In many patients with acute lead poisoning the urinary coproporphyrin output rises again to abnormal levels within a few days after cessation of drug administration. There are two possible interpretations. The edathamil calcium disodium present in the tissues may chelate enough of the remaining tissue lead to restore more normal biochemical pathways in pyrrole metabolism. Because of the rapid urinary excretion of this compound it disappears from the body within a few days. The degree of coproporphyrinuria reappearing at this time might then be a reflection of the residual soft tissue lead concentration which is no longer inactivated by combination with the chelating agent. In such patients subsequent administration of edathamil calcium disodium results again in an increased urinary excretion of lead, the magnitude of which can be predicted by the data in Figure 1. On the other hand, the reduction in coproporphyrin excretion which occurs after five to seven days of edathamil calcium disodium administration might be in part due to direct interference by this chelating agent with the synthesis of porphyrins. It has been reported by Gibson, Neuberger, and Scott (20) that delta-aminolevulinic acid dehydrase is widely distributed in the tissues. This enzyme converts delta-aminolevulinic acid to porphobilinogen, the precursor of the known biologically important porphyrins. These workers found that edathamil inhibits the activity of delta-aminolevulinic acid dehydrase *in vitro* to a greater extent than does lead.

In the convalescent phase of lead poisoning when lead is redistributed among the various tissues and a new, relatively stable, metabolic state has been achieved, the exponential relationship between blood lead concentration and urinary coproporphyrin output shown in Figure 3 can be demonstrated. This finding confirms the correlation found in adult industrial lead workers between blood lead concentration and urinary coproporphyrin excretion reported by Shiels, Palmer, Cornish, and Kearley (7) and Bashour (8). A rather large variation in whole blood lead concentration with respect to urinary coproporphyrin output can be noted in Figure 3. In part, the magnitude of this variation may be explained by the fact that most of the data in Figure 3 were obtained during the first three to six months of convalescence at a time when hemoglobin and hematocrit values were rising rapidly. The resultant variations in total erythrocyte mass may have had an appreciable effect upon whole blood lead concentrations, since in latent lead poisoning approximately 95 per cent of the whole blood lead may be found in the erythrocytes. The magnitude of these variations suggests that the total daily urinary porphyrin excretion reflects more closely the concentration of the "metabolically active" fraction of lead in the tissues responsible for the toxic manifestations than does the whole blood lead concentration. This is particularly pertinent when whole blood lead concentration lies in the range, 0.075 to 0.10 mg. per 100 gm. whole blood, which represents only a moderate increase above the normal value. It is in this range of blood lead concentration that urinary coproporphyrin

excretion may be most useful as a measure of "metabolically active" lead in the body and of the efficacy of treatment of lead poisoning by chelating agents.

The sharp rise in urinary coproporphyrin excretion without concomitant change in whole blood lead concentration seen in convalescing patients during intercurrent acute infections is of interest in view of the clinical observation that such infections have frequently been accompanied by a recrudescence of the clinical manifestations of lead poisoning. Studies (21) in adults without known lead exposure indicate that a two to three-fold increase in urinary coproporphyrin output may occur in chronic bacterial infections. The five to six-fold increments in urinary coproporphyrin output in three of five children with intercurrent infections offer biochemical support to the clinical observation that such infections may intensify the toxic effects of lead in children during the early months of convalescence from acute lead intoxication. When this occurs additional treatment with chelating agents is probably both indicated and efficacious.

Lastly, it should be noted that the method of Schwartz, Zieve, and Watson (10) used for porphyrin analysis in this study measures total urinary coproporphyrin. As pointed out by these authors (22) and confirmed by Comfort, Moore, and Weatherall (23) this method includes the determination not only of pre-formed coproporphyrin but also of significant amounts of an, as yet, unidentified precursor (or precursors) which is quantitatively converted to coproporphyrin during the analysis. Indeed, from his studies Weatherall (24) has suggested that a large portion of the urinary coproporphyrin measured may actually be excreted as precursor. The determination of total coproporphyrin in urine does appear, however, to be a useful measure of "chelatable" lead in the tissues, although preformed coproporphyrin may not be the principle metabolite excreted in the urine in lead poisoning.

SUMMARY AND CONCLUSIONS

In children with lead poisoning a positive correlation has been demonstrated between the urine lead output during the first day following the parenteral administration of edathamil calcium disodium and the total daily urinary coproporphyrin output just prior to the administration of the chelating agent. This relationship is an exponential one and was demonstrated in both the early recovery phase and the chronic phase of plumbism.

A similar correlation was found between the simultaneous daily urinary excretion of coproporphyrin and lead during parenteral edathamil calcium disodium administration.

During the convalescent phase in children who had recovered from acute lead encephalopathy an exponential relation was also found between the whole blood lead concentration and total daily urinary coproporphyrin excretion. This relationship could not be shown during the initial acute phase of encephalopathy when whole blood lead concentration was frequently quite high and decreasing rapidly.

These data indicate that the urinary coproporphyrin output is related to that fraction of lead in the tissues which is available for chelation and urinary excretion under the influence of edathamil calcium disodium. From the urinary coproporphyrin output one can predict the magnitude of lead output in the urine which will follow the parenteral administration of edathamil calcium disodium. Since it is believed that this chelating agent initially removes lead primarily from nonskeletal tissues, it appears reasonable to propose that the urinary coproporphyrin output in children with plumbism is correlated with the concentration of lead in soft tissues. Hence, the urinary coproporphyrin excretion provides a sensitive biochemical index of "metabolically active" and presumably toxic lead in tissues.

ACKNOWLEDGMENTS

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STUDIES ON THE KINETICS OF GLUCOSE UTILIZATION

By C. J. HLAD, Jr., H. ELRICK, and T. A. WITTEN with the technical assistance of A. SMITH, Y. ARAI and T. BOW

(From the Radioisotope Service of the Veterans Administration Hospital and Departments of Medicine and Physiology, University of Colorado Medical Center, Denver, Colo.)

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During recent years quantitative mathematical procedures have been applied to glucose utilization [1] studies in an attempt to replace less exact glucose tolerance tests. Examples of this type of work have been reported by Greville (1), Amatuzio, Stutzman, Vanderbilt, and Nesbitt (2), and Jokipii and Turpeinen (3). The techniques employed by these investigators were somewhat different, but the results indicate that the disappearance of glucose from the blood during glucose infusion or subsequent to a single I.V. glucose load follows an exponential function with respect to time. Greville (1) reported an empirical equation describing the behavior of blood glucose following a sudden glucose load. This equation contained a constant term representing an eventual equilibrium glucose level which was not necessarily equivalent to the initial fasting level. Amatuzio, Stutzman, Vanderbilt and Nesbitt (2) employed a similar equation in which they used the initial fasting level as the constant term. The latter's experimental results indicated that the specific rate constant [2] for glucose utilization was reproducible in the same patient and remained constant for two different glucose loads.

In an effort to avoid the unphysiologic blood glucose levels obtained with sudden glucose loading, Jokipii and Turpeinen (3) used a continuous infusion technique. Their results indicated that various equilibrium levels of blood glucose could be established with different infusion rates; the specific rate constants extracted from their data appeared to be independent of the infusion rate. Furthermore, they implied that the rate constants obtained in this manner may exceed those derived by the single injection technique.

[1] Glucose utilization is defined as the disappearance of blood glucose.

[2] The specific rate constant is a proportionality constant defined as the rate of glucose utilization when the concentration is equal to unity (see appendix).

The equations cited in the above work have been either empirical or have been derived upon the assumption that the disappearance of blood glucose follows a first order chemical reaction. It is the purpose of this paper to derive a kinetic theory of glucose utilization not involving the above assumption but based upon previous experimental work on the relationship between the level of blood glucose and its utilization (4–8). Experimental evidence will be presented to support this theory along with a study of the behavior of the specific rate constant of glucose utilization.

METHODS AND MATERIALS

The subjects employed in this study were males between the ages of 21 and 47, normal with respect to carbohydrate metabolism. The group consisted of resident physicians and hospitalized patients with uncomplicated, non-metabolic illnesses. Each subject received at least 250 gm. of dietary carbohydrate daily for three days before the test, but was fasted 14 hours immediately preceding the test. All subjects were at rest in bed throughout the test. The continuous infusion experiments were not begun until the subject had been at bed rest for 30 to 60 minutes.

Venous blood specimens were obtained without stasis from an indwelling venous cannula in the antecubital vein. This vein carries mixed blood from the deep and superficial tissues of the forearm and hand (9). Capillary blood which contains the same glucose concentration as arterial blood (4) was obtained by finger puncture. All blood samples were obtained at 5 or 10-minute intervals for total test periods ranging from 60 to 180 minutes. Samples were analyzed for glucose by the method of Somogyi-Nelson (10). Care was taken to minimize glycolysis by the use of NaF and refrigeration. Venous samples were analyzed in duplicate and capillary specimens singly. Duplicate venous samples differed by 2.0 ± 1.8 per cent (mean $\pm$ std. dev.)

In the single injection studies, 25 gm. of glucose in 300 ml. of distilled water was injected in 3.5 ± 0.5 minutes. The continuous infusion experiments were performed using a Bowman constant infusion pump. Infusion rates in different subjects varied from 1.5 to 6.0 ml. per min. of 10 per cent glucose solution. In 24 subjects 10 to 15 μc. of Na^{22} was added to the glucose infusion solution. Na^{22}

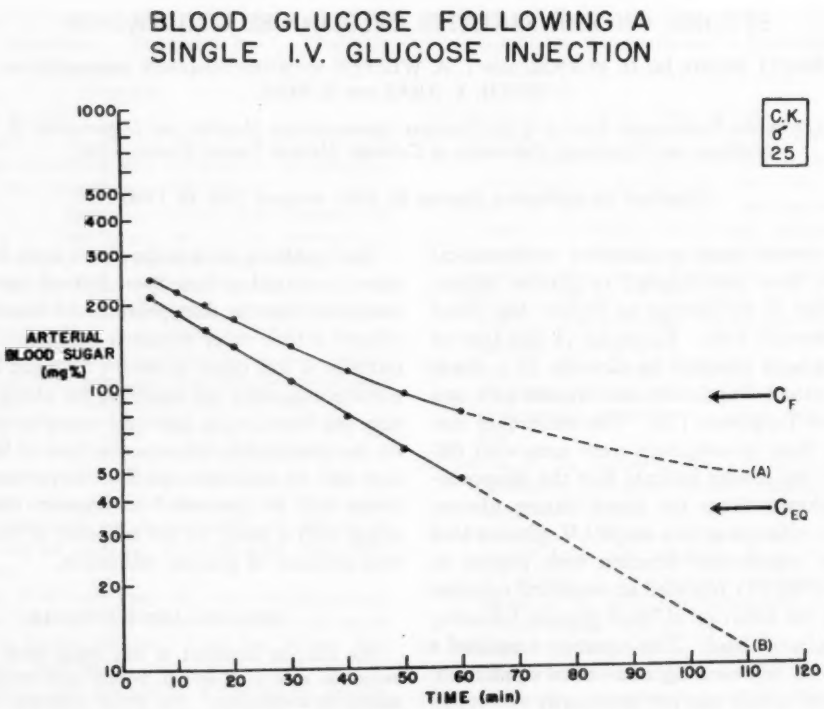

FIGURE 1

Curve (A) represents actual experimental data and is extrapolated to approach an equilibrium level (C_{eq}) defined here as the experimental fasting level.

Curve (B) is a graphical representation of the analysis of these data according to equation *A-5* of the appendix. The ordinate in this case represents the difference between the arterial blood glucose concentration (C_A) and C_{eq}, but has not been shown for reasons of clarity.

The initial fasting level (C_F) is the arterial blood glucose concentration measured just prior to glucose loading.

radioassay on 2 ml. of plasma was accomplished with a R. C. Scientific Scintillation Well Counter.

One hundred and five experiments were done. Of these, 11 were omitted because of poor data. Urine was collected at the end of each experiment and analyzed for total reducing substance by the quantitative Benedict method (11).

RESULTS

A. *Single injection technique*

Twenty-six glucose disappearance curves were obtained from 18 subjects. These curves were analyzed according to the method given in the appendix. An example of this type of experiment with corresponding analysis is shown in Figure 1 and the pertinent results of the analyses of all such experiments are given in Table I. It should be noted in Table I that the mean value of the experimental fasting levels (62.2 ± 26.6) is significantly below ($P = .001$) the mean value of the initial fasting levels (82.2 ± 7.6). Of the individual cases only three values of C_{eq} [3] were well above the corresponding C_F [4]; in three cases these values were approximately equal; and in the remaining cases, C_F exceeded C_{eq}. The specific rate constant for glucose utilization (K) is exemplified in Figure 1 as the slope of curve B. The mean value of the specific rate constants ($.0308 \pm .0120$) as determined here is significantly lower ($P = .001$) than that reported by others ($.0370 \pm .0054$) (2). Also the dispersion of the data is greater. Repeat studies were performed on six

[3] The experimental fasting level (see appendix).

[4] The initial fasting level (see appendix).

subjects, and in none of these was the rate constant reproducible. Urine was collected at the end of each experiment and analyzed for total reducing substance. The mean amount recovered for these subjects was 579 mg.

B. *Continuous infusion technique*

Sixty-nine continuous infusion experiments were done in 63 subjects. The data were analyzed according to the method described in the appendix. Curve A of Figure 2 represents an example of this type of experiment and curve B exemplifies the corresponding analysis. As in Figure 1, the slope of curve B is the specific rate constant for glucose utilization (K). Table II contains relevant data from all continuous infusion experiments. The mean value of the specific rate constants ($.0400 \pm .0126$) obtained by this method was significantly higher ($P = .002$) than that computed from the data of the single injection method ($.0308 \pm .0120$), although blood glucose levels in the latter method far exceed those of the former. In two subjects the infusion period was extended to approximately 150 minutes and in both the specific rate constant remained unchanged over the entire period of the experiments. In five subjects studies were repeated on different days and with a single exception rate constant values could not be reproduced.

From the derivation in the appendix it is possible to compute a theoretical initial fasting level (C_F') based upon all the individual measurements of each experiment. A comparison (Table II) between the mean C_F' (89.8 ± 7.0) and the mean C_F (88.3 ± 6.7) revealed no significant difference ($P = .42$).

In another series of experiments, glucose was infused at a constant rate for a 50-minute control period followed by a 40-minute period with the rate of infusion doubled. The latter type of experiment was performed in 17 subjects and the blood glucose level resulting from the doubled infusion rate was approximately 50 per cent above that of the control period. Pertinent data from these experiments are given in Table III and an example with analysis is shown in Figure 3. The striking finding is that the specific rate constant remains unaltered by this procedure; *i.e.*, there is no significant difference ($P = .60$) between the control periods and those in which the rate of infusion was doubled.

In all continuous infusion experiments, arterial and venous glucose determinations were done. These data have been summarized and the median values are presented in graphic form in Figure 4. It should be noted that although the $C_A - C_V$ [5] values are reasonably constant during the control period, there is a significant rise following an increase in the infusion rate.

Urine was collected at the end of each experiment and analyzed for total reducing substance. The mean amount recovered was 314 mg. which is negligible compared to the total amount of glucose infused (30 to 40 grams).

C. *Volume of distribution*

From the theory given in the appendix it is possible to compute the volume of distribution of glucose. In 68 subjects the mean volume was

TABLE I

The results of single glucose injection experiments *

Subject	Age (*years*)	C_F (*mg.%*)	C_{eq} (*mg.%*)	K ($min.^{-1}$)
H. H.	32	92	60	.0243
C. L.	30	80	70	.0185
T. W.	39	84	55	.0199
T. W.	39	80	50	.0227
T. W.	39	92	70	.0290
E. H.	39	82	80	.0221
B. G.	22	83	50	.0320
B. G.	22	86	60	.0482
C. G.	27	62	30	.0325
J. L.	36	85	50	.0155
C. P.	36	71	85	.0248
G. C.	39	92	71	.0511
G. C.	39	97	80	.0272
P. G.	32	72	150	.0370
C. K.	27	87	35	.0290
G. R.	35	93	50	.0194
L. T.	45	76	62	.0493
C. A.	23	75	30	.0187
F. W.	26	73	102	.0400
F. W.	26	72	75	.0321
L. R.	27	81	8	.0175
L. R.	27	83	83	.0467
L. R.	27	88	55	.0281
P. M.	30	91	40	.0220
L. S.	28	79	65	.0309
L. S.	28	82	50	.0610
Mean values		82.2	62.2	.0308
Std. deviation		7.6	26.6	.0120

* C_F—Initial fasting level. C_{eq}—Experimental fasting level. K—Specific rate constant for glucose utilization.

[5] C_A—The arterial blood glucose concentration. C_V—The venous blood glucose concentration (see the appendix).

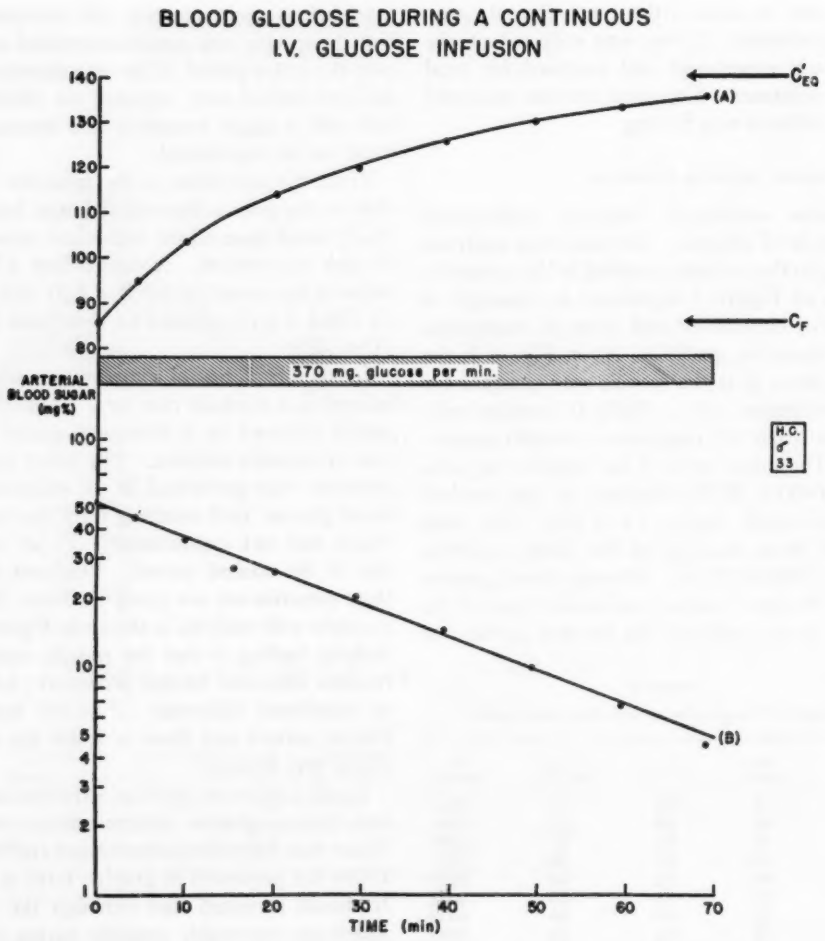

FIGURE 2

Curve (A) represents actual experimental data and is seen to approach an equilibrium level (C_{eq}').

Curve (B) is a graphical representation of the analysis of these data according to equation *B-5* of the appendix. The ordinate in this case represents the difference between the arterial blood glucose concentration (C_A) and C_{eq}'.

The initial fasting level (C_F) is the arterial blood glucose concentration measured just prior to the beginning of glucose infusion.

23.3 ± 6.2 per cent of body weight (Table II). By employing a similar analysis it was possible to compute the value after the rate of glucose infusion had been doubled. This was done in 17 subjects (Table III) and the mean result was found to be 30.8 ± 5.9 per cent of body weight, which is significantly higher ($P = .003$) than the control value of 24.6 ± 5.2 per cent. In other words, an apparent expansion of the available space for glucose distribution occurred subsequent to the increase of the glucose infusion rate.

The above results may have been the result of an increase in extracellular space due to fluid shifts as a consequence of tonicity alteration. This possibility was tested by measuring sodium "space" simultaneously with glucose "space." Na^{22} was

added to the infusion fluid and its space of distribution computed according to a method to be described elsewhere (12). Control spaces were computed on 24 of the 68 subjects. In 11 subjects measurements were obtained before and after doubling the rate of the glucose-Na^{22} infusion. Expressed as percentage of body weight, the mean value of Na^{22} space during the control periods was 21.4 ± 3.4 per cent. The mean value after the increased infusion rate was 22.3 ± 2.7 per cent. There was no significant difference ($P = .40$) between Na^{22} space before and after the glucose infusion rate was doubled.

DISCUSSION

The equation described by Amatuzio, Stutzman, Vanderbilt, and Nesbitt (2) is a close approximation of the empirical equation of Greville (1) and the theoretical equation derived here. The data reported in this paper for the single injection technique fit the latter equation as well as that of Greville. These equations are better satisfied when the experimental fasting level is used rather than the initial fasting level. This is evident from (a) the constancy of K throughout the experimental period in any given subject [6] and (b) the significant difference between the initial and experimental fasting levels in the group of subjects. It would appear that following a rapid glucose load, blood sugar returns to a new level significantly below the initial fasting level (Figure 1). The experimental fasting level is a term of the continuous function (equation *A-5*, appendix)

[6] The data was also analyzed by plotting the logarithm of the difference between the arterial glucose and the initial fasting level *vs.* time (Amatuzio, Stutzman, Vanderbilt, and Nesbitt, [2]) and by plotting the log of the arterial glucose *vs.* time (Bastenie, Conard, and Franckson [13]). By either method a constant K may be approximated during the initial experimental period; however, the constancy of K cannot be maintained throughout the complete experimental period.

TABLE II

The results of continuous glucose infusion experiments *

Subject	Age (*years*)	Weight (*kg.*)	$R_I C_I$ (*mg./min.*)	C_F (*mg. %*)	C_F' (*mg. %*)	C_{eq}' (*mg. %*)	K ($min.^{-1}$)	Glucose space %
G. N.	35	65.9	300	94	96	155	.0300	25.7
H. C.	33	71.8	295	81	81	112	.0533	24.5
C. L.	29	65.9	284	81	82	127	.0478	19.8
C. R.	31	69.1	300	90	90	168	.0277	20.0
C. F.	45	74.1	317	101	102	185	.0248	20.8
P. H.	30	60.0	270	96	95	166	.0260	24.4
R. W.	27	56.4	250	84	83	115	.0520	26.7
F. R.	32	99.6	330	87	87	135	.0307	22.6
J. D.	36	66.6	360	83	85	130	.0310	38.8
H. R.	41	94.1	310	92	92	139	.0336	21.2
A. S.	22	67.3	280	86	86	130	.0340	29.7
B. M.	47	71.7	300	89	93	139	.0317	28.7
J. C.	28	64.4	340	95	98	145	.0420	26.7
R. W.	35	83.6	250	81	80	112	.0639	14.5
E. A.	27	69.3	310	84	83	128	.0399	21.1
T. B.	35	71.1	300	84	83	116	.0730	17.5
C. H.	35	77.3	305	81	89	144	.0435	16.4
V. F.	37	65.0	290	80	81	130	.0335	27.2
V. B.	31	70.4	285	87	98	150	.0310	25.1
C. B.	37	58.9	345	83	80	191	.0260	20.3
B. D.	40	56.7	300	89	85	120	.0470	32.2
W. D.	27	85.4	350	85	85	155	.0333	17.4
K. R.	21	87.7	360	77	89	143	.0265	28.7
G. N.	35	67.4	295	110	113	170	.0340	22.4
C. C.	46	71.9	192	90	89	128	.0200	34.1
F. R.	32	100.0	260	79	80	103	.0825	13.4
J. D.	36	66.6	300	82	85	112	.0530	30.9
R. B.	47	69.8	320	93	87	130	.0313	33.7
L. T.	31	60.4	310	89	90	133	.0565	21.3
R. R.	29	76.8	270	95	100	135	.0295	34.2
R. T.	30	60.4	275	91	91	129	.0430	27.8
D. D.	36	77.7	300	87	87	135	.0320	25.1
G. W.	32	78.2	320	79	77	130	.0294	26.0
R. G.	36	69.7	360	89	89	136	.0557	19.5
R. B.	29	81.6	360	88	93	145	.0395	21.5

TABLE II—*Continued*

Subject	Age (*years*)	Weight (*kg.*)	R_IC_I (*mg./min.*)	C_F (*mg. %*)	C_F' (*mg. %*)	C_{eq}' (*mg. %*)	K ($min.^{-1}$)	Glucose space %
C. J.	26	70.7	370	86	86	165	.0238	27.8
E. J.	25	79.0	360	86	89	132	.0404	26.2
J. P.	31	65.0	360	80	86	154	.0303	26.7
W. Y.	22	77.8	344	88	93	140	.0390	23.9
G. M.	22	60.0	365	87	86	173	.0333	21.0
W. F.	32	53.2	325	75	86	144	.0263	33.7
H. C.	33	71.8	370	86	98	140	.0330	30.0
L. S.	24	64.6	360	86	90	135	.0412	30.1
F. A.	22	67.1	220	80	89	118	.0475	23.8
L. M.	22	60.9	330	83	79	149	.0470	16.5
V. O.	29	65.0	290	81	82	133	.0495	17.7
H. E.	29	65.5	320	99	98	185	.0331	17.0
L. D.	33	63.1	300	88	92	145	.0395	22.5
R. S.	32	71.1	300	92	93	165	.0394	14.8
P. W.	21	66.8	270	101	101	146	.0354	25.1
W. H.	24	58.6	300	84	84	150	.0532	14.6
J. C.	32	70.5	295	93	92	160	.0591	10.4
J. H.	21	61.1	270	93	95	143	.0406	22.7
A. M.	27	87.7	250	91	91	122	.0310	29.5
E. R.	23	75.7	310	90	89	153	.0285	22.4
W. D.	36	69.6	277	89	89	140	.0442	17.5
A. T.	39	68.2	320	101	102	175	.0196	32.6
J. S.	38	79.1	300	95	97	170	.0465	11.1
J. G.	26	53.9	275	90	90	147	.0394	22.7
R. R.	29	75.0	290	95	103	145	.0298	30.7
J. C.	34	80.5	290	90	90	143	.0447	15.2
E. H.	37	73.3	270	92	93	132	.0604	15.5
T. D.	37	68.4	265	106	105	160	.0532	13.2
A. O.	43	73.6	320	89	87	140	.0266	30.8
M. A.	34	67.8	390	97	98	180	.0372	18.9
C. R.	23	71.1	310	82	80	145	.0366	18.3
F. R.	29	60.5	275	87	91	132	.0688	16.1
C. G.	30	60.5	270	83	84	115	.0526	27.6
R. B.	47	69.8	300	84	88	132	.0410	23.9
Mean values				88.3	89.8		.0400	23.3
Std. deviation				6.7	7.0		.0126	6.3

* R_I—Rate of infusion (ml./min.). C_I—Concentration of infused fluid (mg./ml.). C_F—Initial fasting level. C_F'—Theoretical initial fasting level. C_{eq}'—Blood glucose level at equilibrium ($t = \infty$). K—Specific rate constant for glucose utilization. Glucose space—The volume of distribution of glucose expressed as per cent of body weight.

describing glucose disappearance from a loaded system. This level serves as reference level for a new physiologic state following the glucose load. It should be noted that this new state is apparently steady throughout the disappearance phase of blood glucose, but that it is different from the basal state. The fact that the new level was usually below the initial level is compatible with the hypoglycemia commonly observed following rapid glucose loading. It is also common knowledge that blood sugar will eventually return to the initial fasting level following the hypoglycemic phase. Thus it is apparent that the new state created by the glucose load is not persistent. It should be emphasized that although equation *A-5* describes blood glucose to the minimum hypoglycemia, it is not intended to describe the return phase which represents a change in the state of the system.

In contrast to the sudden elevation of blood glucose resulting from a single glucose injection, blood glucose levels were increased gradually with the continuous infusion method. The equation (*B-5*) given here is well satisfied by the experimental data. In the continuous infusion experiments there was no significant difference between the initial fasting level and the computed initial fasting level. This computed level is derived from all the measurements of each experiment. Since these levels are statistically identical a more stable state is apparently maintained throughout continuous glucose infusion, resulting in less disturbance to the glucose regulation mechanisms. It is probable that the rate constant derived by the continuous infusion method is more characteristic of the basal physiologic state.

There is considerable evidence in animals that

hyperglycemia resulting from glucose loading, stimulates endogenous insulin release (14–16). However, it is difficult to compare these experiments to the present ones since the former were done at much higher blood sugar levels. Recently, it has been shown in humans by direct insulin assay that alimentary hyperglycemia results in a mean increase of 600 per cent in plasma-insulin activity one hour after the ingestion of 50 gm. of glucose (17). Using the same authors' data, this

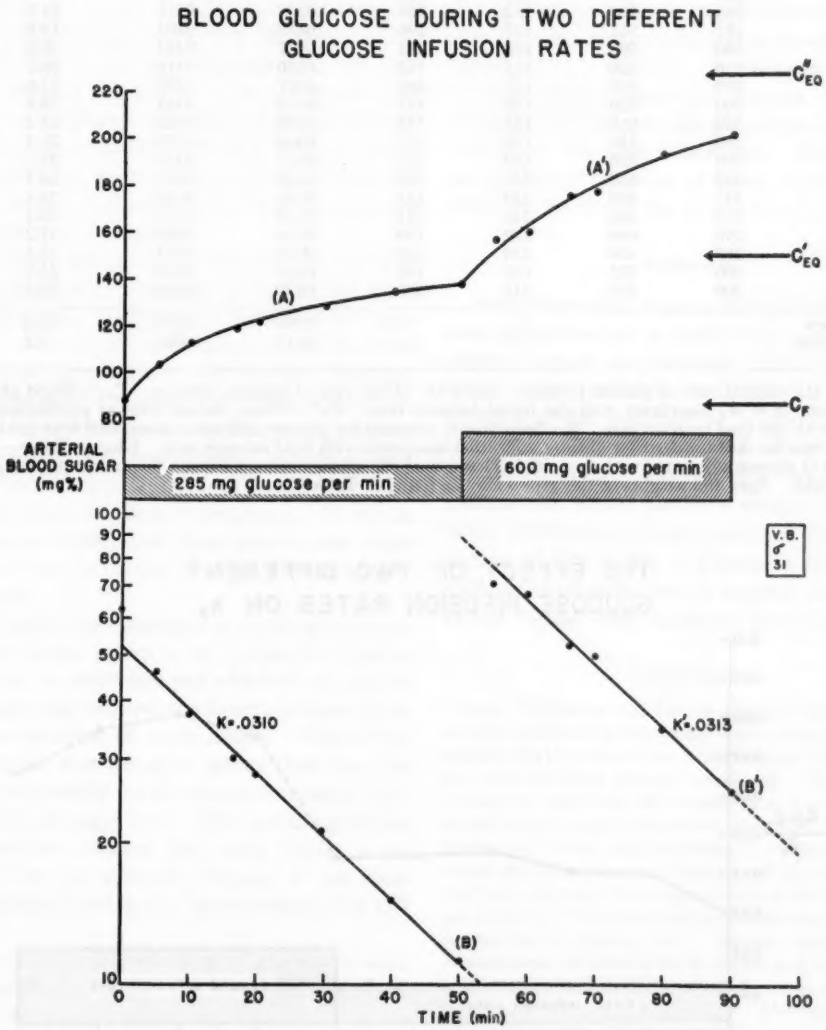

FIGURE 3

Curves A and A′ represent actual experimental data with their respective equilibrium levels at C_{eq}' and C_{eq}''.

Curves B and B′ represent the analysis of these data according to equation *B-5* of the appendix. The ordinate in this case represents the difference between the arterial blood glucose concentration (C_A) and the respective equilibrium level.

The initial fasting level (C_F) is the arterial blood glucose concentration measured just prior to glucose infusion.

TABLE III

The results of continuous glucose infusion experiments with elevated infusion rates *

Subject	R_IC_I (1) (mg./min.)	R_IC_I (2) (mg./min.)	C_{eq}' (mg. %)	C_{eq}'' (mg. %)	K ($min.^{-1}$)	K' ($min.^{-1}$)	Glucose space (1) %	Glucose space (2) %
G. N.	300	580	155	240	.0300	.0297	25.7	29.3
H. C.	295	500	112	164	.0533	.0510	24.5	24.6
C. L.	284	567	127	196	.0478	.0405	19.8	28.9
C. R.	300	593	168	255	.0277	.0284	20.0	28.2
R. W.	250	500	115	165	.0520	.0510	26.7	34.4
F. R.	330	590	135	200	.0307	.0288	22.6	27.4
J. D.	360	620	130	187	.0310	.0344	38.8	40.7
H. R.	310	610	138	195	.0336	.0360	21.2	28.6
A. S.	280	550	129	203	.0340	.0279	29.7	36.2
B. M.	300	560	139	216	.0317	.0323	28.7	28.5
J. C.	340	630	145	220	.0420	.0425	26.7	29.0
E. A.	310	600	130	185	.0399	.0363	24.1	37.9
C. H.	305	600	144	222	.0435	.0425	16.4	22.2
V. F.	290	600	130	190	.0335	.0355	27.2	40.0
V. B.	285	600	150	228	.0310	.0313	25.1	30.4
R. B.	300	595	132	185	.0410	.0410	23.9	36.2
T. B.	300	590	116	180	.0730	.0550	17.5	20.8
Mean values					.0397	.0379	24.6	30.8
Std. deviation					.0117	.0086	5.2	5.9

* R_IC_I (1)—Initial rate of glucose infusion. R_IC_I (2)—Final rate of glucose infusion. C_{eq}'—Blood glucose level at equilibrium ($t = \infty$) associated with the initial infusion rate. C_{eq}''—Blood glucose level at equilibrium ($t = \infty$) associated with the final infusion rate. K—Specific rate constant for glucose utilization associated with initial infusion rate. K′—Specific rate constant for glucose utilization associated with final infusion rate. Glucose Space—Volume of distribution of glucose associated with the initial (1) and final (2) infusion rates. These values are expressed as per cent of body weight. Ages and weights of this group may be found in Table II.

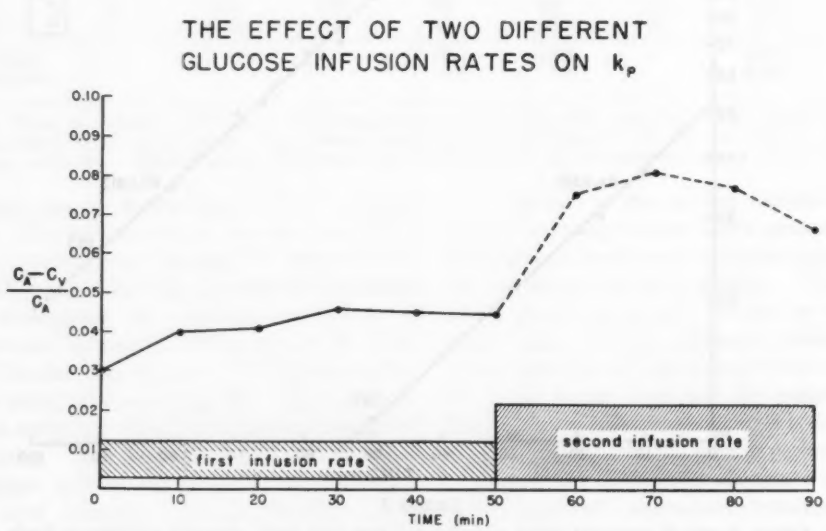

FIGURE 4

The ordinate ($C_A - C_V/C_A$) is shown in the appendix to be a measure of the specific rate constant (k_p) for peripheral glucose utilization and represents the arterio-venous glucose difference divided by the arterial blood glucose concentration. The plotted data represent median values determined from 17 experiments.

is equivalent to the addition of 4.8 units of insulin in an average man. It has been reported (18) and substantiated by work in this laboratory (19) that small amounts (2.5 to 4 units) of exogenous insulin will cause a striking increase in the specific rate constant. It was, therefore, surprising to observe that the mean specific rate constant obtained from the sudden loading technique is significantly lower than that obtained from the continuous infusion technique. It would appear that of the two methods for adding glucose to the system, the sudden loading technique with its resulting higher blood sugars is more likely to stimulate insulin release and thus more likely to increase the specific rate constants.

With the constant infusion technique it was possible to determine whether or not an elevated blood sugar alters the specific rate constant in the same patient. The results of the experiments in which the glucose infusion rate was doubled following the control period showed that no significant difference exists between the specific rate constants of the control and experimental periods (Table III). Furthermore, K remained constant throughout each phase of these experiments. If insulin secretion is a function of blood glucose, one might expect an ever increasing K value with rising glucose levels.

Thus, under the conditions of these experiments, by either single injection or continuous infusion of glucose, no evidence was obtained to support the concept that intravenous hyperglycemia stimulates the secretion of extra insulin. These findings suggest that the liver rather than the pancreas is responsible for the minute-to-minute regulation of blood sugar level. They are in agreement with previous evidence that extra insulin is not required for the adequate disposal of an intravenous glucose load in the depancreatized dog (20, 21).

The volume of distribution of glucose is obviously complex. Possibly the best definition of this "space" as measured here is that which is occupied by "exchangeable" glucose. The apparent increase in glucose "space" with elevated blood glucose is not explained by a simple fluid shift, since Na^{22} space remained constant. One might speculate that since the specific rate constant is unaltered, and Na^{22} space remains constant, the observed increase in glucose "space" is a change associated with increased cellular metabolism of glucose.

A derivation is given in the appendix which shows that $(C_A - C_V/C_A)$ is proportional to the specific rate constant of peripheral glucose utilization (k_p) under certain conditions. An examination of Figure 4 shows that elevation of the glucose infusion rate results in an elevation of this variable $(C_A - C_V/C_A)$. It has been shown that the total disappearance rate constant (k) remains unaltered throughout this procedure and that the volume of distribution increases. This would be the predicted behavior of these variables to account for the rise in the $(C_A - C_V/C_A)$ values.

SUMMARY

Some theoretical aspects of the kinetics of glucose utilization and a method for computation of glucose "space" are described. One hundred five studies were performed in 81 normal men using either a single injection or continuous infusion technique. Data are presented which support the derived equations. No evidence was obtained to indicate that excess insulin is released in response to the intravenous administration of glucose. The volume of distribution of glucose is discussed and evidence is presented which suggests an expansion of this "space" with increased blood sugar levels.

APPENDIX

Lang, Goldstein, and Levine reported that peripheral glucose utilization in the normal dog is a linear function of arterial blood glucose over a wide range (79 to 645 mg. per cent) of blood glucose values (6). The data were obtained in what may be considered as a steady physiological state (constant infusion of glucose). Prior to this, Soskin and Levine had established a proportionality between peripheral glucose utilization and blood sugar which was linear between blood sugar levels of 100 and 400 mg. per cent (5). The latter data were obtained in the eviscerated dog in a steady state. Somogyi obtained careful measurements of arterial blood sugars and corresponding arteriovenous differences in man before and after the oral ingestion of 100 gm. of glucose (4). Although blood flow was not measured, there was a linear correlation between A−V difference and arterial blood level during the disappearance phase of blood glucose.

This evidence indicates that in both steady and quasi-steady states, glucose utilization is a linear function of arterial blood glucose over a wide range of blood glucose levels. The range of blood glucose values in the present study is within the range over which linearity has been demonstrated. The linear function described above may

be expressed as follows:

$$U_P = k_p \cdot G_A, \qquad (1)$$

where U_P represents the rate of peripheral utilization of glucose; k_p the specific rate constant associated with peripheral utilization; and G_A the total glucose presented to the peripheral cells for metabolism at any time.

The rate of peripheral utilization of glucose may also be expressed as:

$$U_P = R(C_A - C_V). \qquad (2)$$

where R is the arterial blood flow rate and C_A and C_V are the arterial and venous glucose concentrations, respectively. Combining equations *1* and *2* and solving for k_p results in:

$$k_p = \frac{C_A - C_V}{C_A} \cdot \frac{R}{V}, \qquad (3)$$

where V is the volume of distribution of G_A. It should be noted that if R and V remain constant, k_p is proportional to $\frac{C_A - C_V}{C_A}$.

Soskin, Essex, Herrick, and Mann (7) showed that liver uptake or output of glucose is related to the arterial glucose level. This work was done in the dog using both a single injection and a continuous infusion of glucose. By replotting their data it is possible to show that liver uptake or output of glucose is a linear function of arterial blood glucose. Bondy, James, and Farrar reported similar results in man (8). Analysis of their data reveals that a linear correlation exists between the mean values of A−V difference across the liver and mean values of arterial blood glucose. Thus it appears that in the dog and the human the liver uptake or output of glucose can be considered to be a linear function of arterial glucose level. This function may be expressed as follows:

$$U_L = -mG_A + b, \qquad (4)$$

where U_L represents the rate of uptake or output of glucose by the liver ($U_L > 0$; output $U_L < 0$; uptake); and m and b are constants characteristic of the state of the system. With these two equations it is possible to derive ideal relationships which govern the behavior of blood glucose either subsequent to sudden glucose loading or during the continuous infusion of glucose.

A. *Sudden glucose injection*

Previous work in man indicates that blood glucose disappearance after a single intravenous glucose load follows an exponential function shortly after the peak blood glucose level (1, 2, 9). In these experiments adequate mixing is apparently obtained within 10 minutes after the glucose injection. This function also implies that the system does not deviate too far from a steady state during glucose disappearance. At fasting equilibrium when the blood sugar level is constant, it may be stated that:

$$U_L \geqslant 0, \quad U_P \geqslant 0 \quad \text{and} \quad U_L = U_P.$$

But following a sudden glucose load this equilibrium is upset and the liver responds in such a manner that:

$$U_L < 0 \quad \text{and} \quad U_P > 0$$

then, subsequent to adequate mixing:

$$\frac{dG_A}{dt} = U_L - U_P = -mG_A + b - k_pG_A = -KG_A + b, \qquad (A\text{-}1)$$

where K represents the specific rate constant for glucose disappearance from the blood and is associated with the total utilization of glucose. Upon integration:

$$[\ln (b - KG_A)]_{G_0}^{G_A} = [-Kt]_0^t, \qquad (A\text{-}2)$$

where G_0 is the value of G_A at $t = 0$ which is defined at the point where mixing is complete. Evaluation of *A-2:*

$$b - KG_A = (b - KG_0)\, e^{-Kt} \qquad (A\text{-}3)$$

$$\text{At } t = \infty, \quad G_A = G_{eq}, \quad \text{and} \quad \frac{b}{K} = G_{eq}$$

G_{eq} represents the theoretical glucose content at $t = \infty$. Upon substitution *A-3* becomes:

$$G_A - G_{eq} = (G_0 - G_{eq})\, e^{-Kt}. \qquad (A\text{-}4)$$

In concentration form for a constant volume of distribution, *A-4* becomes:

$$C_A - C_{eq} = (C_0 - C_{eq})\, e^{-Kt}, \qquad (A\text{-}5)$$

where C_A is the arterial blood glucose concentration at any time (t); C_{eq} is equilibrium blood glucose level and should be equivalent to the initial fasting level (C_F) providing the state of the system remains stable as a consequence of glucose loading; and C_0 is the concentration associated with G_0. It should be noted that a plot of $C_A - C_{eq}$ versus t on semilogarithmic paper results in a straight line with a zero intercept of $C_0 - C_{eq}$. This is exemplified by curve B of Figure 1.

B. *Continuous glucose infusion*

In the non-equilibrium state during continuous glucose infusion,

$$R_IC_I + U_L > U_P,$$

where R_I is the rate of infusion and C_I is the infused glucose concentration. Then:

$$\frac{dG_A}{dt} = R_IC_I - mG_A + b - k_pG_A = R_IC_I + b - KG_A. \qquad (B\text{-}1)$$

Upon integration:

$$[\ln (R_IC_I + b - KG_A)]_{G_F'}^{G_A} = [-Kt]_0^t, \qquad (B\text{-}2)$$

where G_F' is the value of G_A at $t = 0$ and is theoretically equivalent to the glucose content at fasting equilibrium preceding glucose infusion. Evaluating *B-2:*

$$R_IC_I + b - KG_A = (R_IC_I + b - KG'_F)\, e^{-Kt} \qquad (B\text{-}3)$$

$$\text{At } t = \infty, \quad G_A = G'_{eq} \quad \text{and} \quad b = KG'_{eq} - R_IC_I,$$

where G'_{eq} is the equilibrium glucose content at $t = \infty$ and after substitution, *B-3* becomes:

$$G'_{eq} - G_A = (G'_{eq} - G'_F)\, e^{-Kt}. \qquad (B\text{-}4)$$

In concentration form, *B-4* becomes:

$$C'_{eq} - C_A = (C'_{eq} - C'_F)\, e^{-Kt}, \qquad (B\text{-}5)$$

where C'_{eq} is the eventual equilibrium glucose level at $t = \infty$ and C'_F is the theoretical initial fasting level. It

should be noted that a plot of $C'_{eq} - C_A$ versus t on semilogarithmic paper results in a straight line with a zero intercept of $C'_{eq} - C'_F$. This is exemplified by curve B of Figure 2. Furthermore, C_F' is observable after the zero intercept is established.

C. *Volume of distribution*

Within the limits defined here, the slope of equation *B-5* is a maximum at $t = 0$ and a minimum at $t = \infty$. The maximum slope (S_{max}) may be evaluated as:

$$S_{max} = K(C'_{eq} - C'_F). \qquad (C\text{-}1)$$

If a negligible volume of glucose is infused into a system of constant volume and no process takes place other than distribution of the infused glucose, the concentration within the constant volume will increase as a linear function of time. The slope of this function is equivalent to $\frac{R_I C_I}{V}$. Since the infused glucose is the only exogenous glucose added to the system and since $U_L \geqslant 0$ only at fasting equilibrium, it may be assumed that the maximum change in blood glucose results from the infused glucose. Thus the slope due to infusion alone is the limiting value of the slope of equation *B-5*. Therefore:

$$\frac{R_I C_I}{V} = K(C_{eq}' - C_F') \qquad (C\text{-}2)$$

and solving for V:

$$V = \frac{R_I C_I}{K(C_{eq}' - C_F')}.$$

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SOME STUDIES ON THE NATURALLY OCCURRING LEUCOCYTE AGGLUTININS[1, 2]

By JOHN J. BUTLER

(From St. Mary's Hospital, Rochester, N. Y.)

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The purpose of this report is to describe some investigations which attempt to define the nature and incidence of naturally occurring leucocyte agglutinins.

Agglutinins have been demonstrated in the sera of patients with acquired hemolytic anemia (1) and thrombocytopenic purpura (2, 3) and these have been distinguished from naturally occurring anti-red cell (4) and anti-platelet (5) agglutinins. Leucocyte agglutinins have been demonstrated in various clinical conditions including lupus erythematosus (6), aleukemic leukemia (6), pancytopenia (6, 7), agranulocytosis due to pyramidon (8) and chronic neutropenia (9). Some have implicated these agglutinins as the causative factors for the leukopenia in these diseases.

Goudsmit and van Loghem (6) tested the sera of 100 healthy donors against type O white cells. They found weakly positive clumping reactions in 10 instances. Dausset, Nenna, and Brecy (10) studied the sera of 300 normal donors for the presence of agglutinins to compatible leucocyte suspensions and failed to demonstrate any. Dausset (11) cited some unpublished experiments in collaboration with van Loghem in which combinations of 50 normal sera and 50 corresponding varieties of white cells were tested for leucocyte agglutination. They observed frequent but inconstant agglutinations in the combinations which were incompatible for the ABO blood groups. Moeschlin and Schmid (12) tested blood samples of 30 persons in 380 leucocyte serum combinations without regard for A, B and O blood groups. They noted leucocyte agglutination in 60 cases of the 380 combinations. Ninety-seven per cent of these agglutinations occurred in leucocyte serum combinations which were incompatible with respect to the A, B, and O, blood groups and they concluded that this agglutination was caused by the blood group specific iso-agglutinins. This inference was made in spite of the fact that leucocyte agglutination was absent in two-thirds of the combinations in which it would have been expected on the basis of intensive erythrocyte agglutination. They thought that it was improbable that a low serum titer of group specific erythrocyte agglutinins could be responsible for the absence of leucocytic agglutination.

METHODS

Plasma was handled with sterile technique and was stored at $-20°$ C until used. Sequestrene was used as an anticoagulant throughout. All glassware was siliconized and the metal parts were coated with arquad each time the equipment was used. The silicone was stripped from the glassware after each usage by soaking in 10 per cent Na OH solution. Blood was collected for the preparation of white cells in the following manner: A needle was inserted in the antecubal vein and blood was allowed to flow up to the mark of a 15-ml. centrifuge tube containing 1 ml. of 1 per cent sequestrene and 1.25 ml. of Dextran having an intrinsic viscosity greater than .41. The centrifuge tube was covered with parafilm and inverted gently three times. The blood was allowed to settle for about 30 minutes and the supernatant was removed. The white cell counts were usually between 10,000 and 15,000 per cu. mm. This plasma contained platelets and about equal numbers of red cells and white cells. The plasma samples and leucocyte suspensions were adjusted to pH 6.9 by the addition of 1/9 N citric acid. Methyl orange was used as an indicator and about 1 drop of acid was required for each .4 ml. of plasma and about .3 ml. of acid for each 7 ml. of leucocyte suspension.

When a diluent was required in preparing serial dilutions, a non-reacting type A plasma of low titer was used after it had been absorbed with type B cells.

Test for leucocyte agglutinins. This test was performed by mixing .15 ml. of white cell suspension with .40 ml. of test plasma and one drop of 30 per cent bovine albumin in a 10×75 mm. serological test tube. This was incubated in a water bath at 37° C and read after two

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hours. Each test for leucocyte agglutination was performed on at least two and in some cases three different occasions. The leucocytes were resuspended after incubation by shaking the test tube quickly about six times. A drop was poured on a glass slide and allowed to flow out in a thin layer by tilting the slide. The slide was studied for white cell clumping under the low power of the microscope either with reduced light or with phase microscopy.

Erythrophagocytic index. After the result of the test for leucocyte agglutinins was read microscopically, there was a considerable portion of the test material left in the serological test tube. This was allowed to resettle for two hours, and dry smears were made of the sediment on cover slips which were stained with Wright's stain. Five hundred white cells were counted and the number of leucocytes which had ingested red cells was noted. The percentage which contained red cells was designated the erythrophagocytic index (13). An index of two or above was arbitrarily designated as significant.

Test for inhibition by specific polysaccharide of blood group A. The naturally occurring leucocyte agglutinins were tested for inhibition by adding one drop of specific polysaccharide of blood group A reagent [3] to .4 ml. of the type O or type B plasma which was to be tested. This was incubated for 30 minutes at 25° C before being added to the leucocyte suspension as in the test for leucocyte agglutinins. A positive control test for leucocyte agglutinins contained in the type O or type B plasma was always run. A second control consisted of one drop of specific polysaccharide of blood group A reagent being incubated with the type O or type B plasma before addition of a 2 per cent suspension of type A red cells in saline.

Test for the potency of the specific polysaccharide of blood group A reagent. Equal volumes of this substance were added to the serial dilutions of anti A blood grouping plasma and were incubated for 30 minutes at 25° C before the type A test cells were added. The specific polysaccharide reagent was found to inhibit the anti-A blood grouping plasma at a titer of 1:8.

Test for salivary antigens. Saliva was tested for the presence of A and B substances as follows: The saliva in a test tube was placed in a boiling water bath for 20 minutes. It was then centrifuged and the clear supernatant liquid was diluted serially with saline. One-tenth ml. of this material was added to .1 ml. of anti A or anti B typing sera and allowed to stand at room temperature for 30 minutes. Then, .1 ml. of a 2 per cent suspension of A or B cells was added. After 30 minutes the tubes were centrifuged and examined for agglutination. A control tube contained no saliva and another control contained saliva of a known secretor. In the first control there was a 4+ agglutination and the same was found in all dilutions of the unknown when they were non-secretors. Secretors were defined as those whose saliva inhibited agglutination in a dilution of 1:8. The typing sera was adjusted with saline so that a titer of 1:128 produced a trace of clumping when tested against the appropriate red cells.

[3] Kindly furnished for use by Sharpe and Dohme Co.

TABLE I

Protocol and results of a positive test for the presence of a leucocyte agglutinin inhibitor in saliva

TEST PLASMA ML.	SALIVA ML	CONTROL PLASMA ML	CELLS ML	RESULT AGGLUTINATION
.40	—	—	.15	++++
.394	.006	—	.15	0
.388	.012	—	.15	0
.375	.025	—	.15	0
.35	.05	—	.15	0
.30	.10	—	.15	0
.20	.20	—	.15	0
—	—	.40	.15	0
.394	—	.006	.15	++++
.388	—	.012	.15	++++
.375	—	.025	.15	++++
.35	—	.05	.15	+++
.30	—	.10	.15	++
.20	—	.20	.15	+

Test for salivary inhibition. The saliva of the white cell donor which was tested for its ability to inhibit the leucocyte agglutinin was heated for 30 minutes at 100° C to destroy any enzymes present. This saliva was then serially diluted with the plasma containing the agglutinin before being added to the leucocyte suspension and albumin as in the test for leucocyte agglutinins. The effect of dilution was controlled by substituting a non-reacting plasma for the saliva. Table I illustrates an example of complete inhibition of an agglutinin by a white cell donor's saliva.

Test for hemolysins. Hemolysins were tested for in the following manner: The serum to be tested was inactivated at 56° C for thirty minutes after which .5 ml. portions of serial 2 fold dilutions in .85 per cent sodium chloride were added to each of nine tubes. Normal saline was added to the tenth tube which served as a control. One-tenth ml. of fresh, normal, unheated AB serum (diluted with an equal part of saline) and .5 ml. of a 3 per cent saline suspension of thrice washed test cells were added to each of the ten tubes, which were then shaken. The mixtures were placed in a water bath at 37° C for thirty minutes, centrifuged for one minute at 1,500 RPM and examined for the presence of hemolysis.

Absorption of the C antibody of group O plasma. Plasma from a group O donor was absorbed with group B cells. The cells used for absorption were washed three times with normal saline solution and then packed. One volume of plasma was mixed with one volume of these cells and placed in a water bath at 37° C for one-half hour, then allowed to stand at room temperature for one-half hour, then centrifuged and the plasma re-

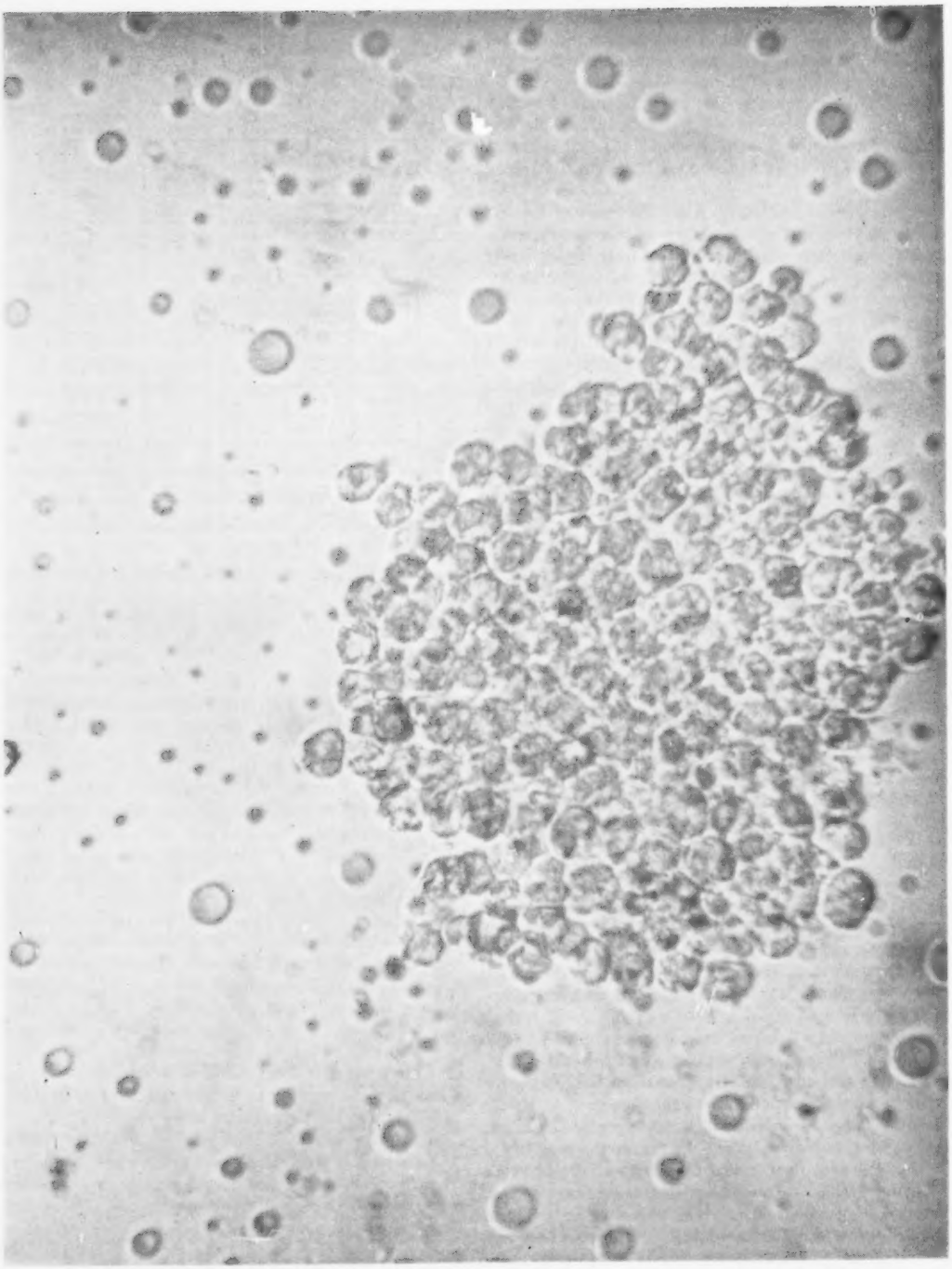

FIG. 1. PHOTOMICROGRAPH UNDER HIGH DRY MAGNIFICATION SHOWING A CLUMP COMPOSED ENTIRELY OF LEUCOCYTES

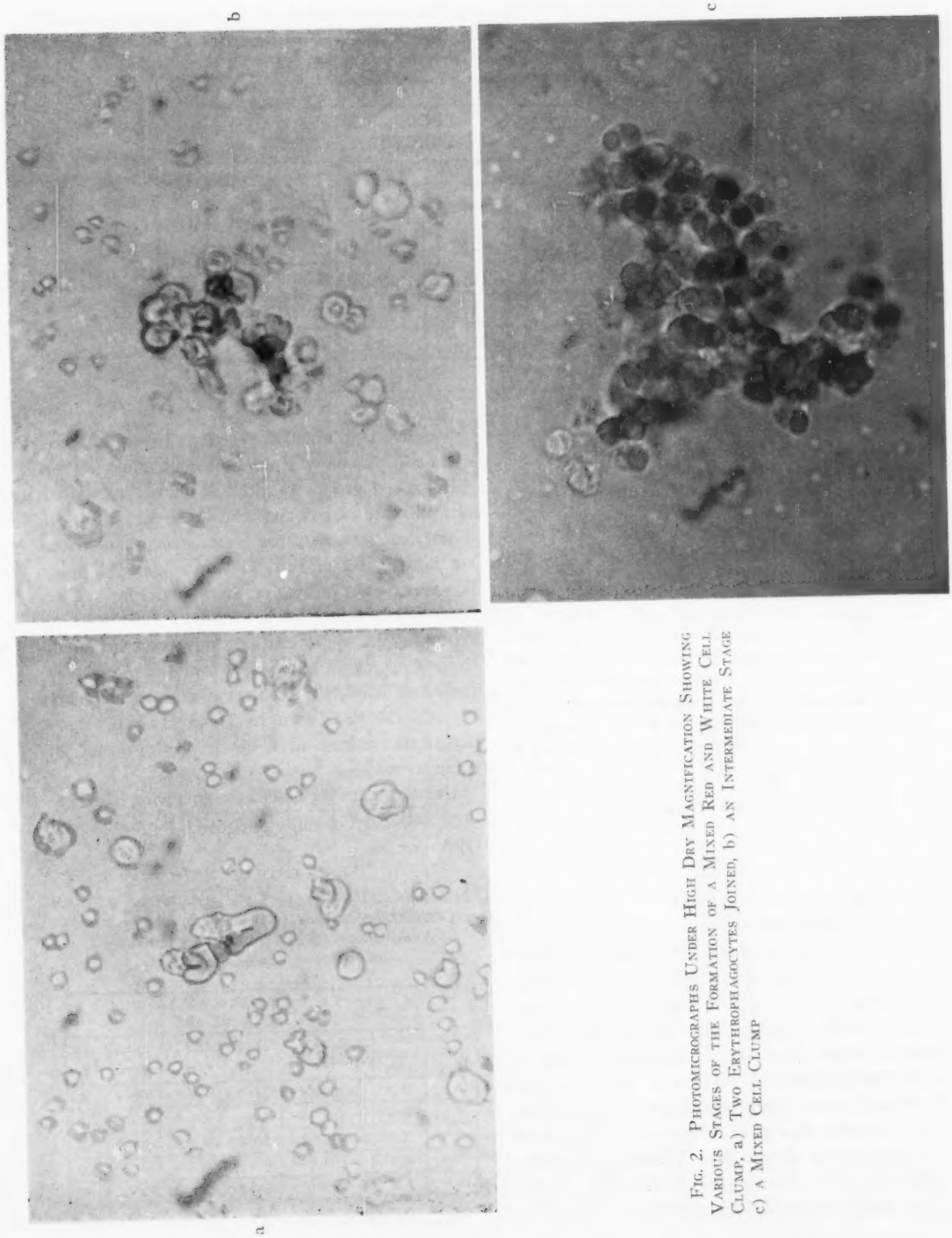

FIG. 2. PHOTOMICROGRAPHS UNDER HIGH DRY MAGNIFICATION SHOWING VARIOUS STAGES OF THE FORMATION OF A MIXED RED AND WHITE CELL CLUMP, a) TWO ERYTHROPHAGOCYTES JOINED, b) AN INTERMEDIATE STAGE c) A MIXED CELL CLUMP

TABLE II

The results of tests for leucocyte agglutination (A) and erythrophagocytosis (E), (as measured by the erythrophagocytic index) repeated for four successive days are compared

			REPRODUCIBILITY ON SUCCESSIVE DAYS							
PLASMA DONOR TYPE	CELL DONOR TYPE	PLASMA DONOR ANTI-B TITER	DAY 1		2		3		4	
			A	E	A	E	A	E	A	E
O−	B +	1:256	2+	26	2+	13.6	3+	0	2+	10.4
O−	B +	1:64	2+	42.8	3+	25.2	2+	10.4	2+	24.4
O+	B +	1:256	2+	15.2	2+	8.8	1+	0	1+	6.0
A+	B +	1:128	3+	0	2+	0	1+	0	−	0
A+	B +	1:256	4+	0	2+	0	−	0	−	0

moved. This plasma was then tested for leucocyte agglutinins and erythrophagocytosis against white cell suspensions from group A donors.

Controls of unabsorbed plasma from the same group O donor were tested simultaneously.

Test for the ability of plasma to cause erythrophagocytosis in the presence of incompatible red cells. Red cells which were incompatible with respect to the ABO group of the plasma being tested were washed three times in large quantities of physiological saline. One-half ml. of a 2 per cent suspension of these red cells was centrifuged and the supernatant was discarded. One-half ml. of a leucocyte suspension in plasma collected in the usual manner was added to the sedimented red cells. These were resuspended by gentle agitation and incubated for two hours at 37° C. This preparation was examined for clumping; and, after resettling for one hour, smears of the sediment were made. These smears were stained with Wright's stain and examined for erythrophagocytosis.

RESULTS

To date, a total of 750 tests for leucocyte agglutination have been performed, and the erythrophagocytic index has been determined on 450 of these. It was noted in this series that positive agglutination reactions involving leucocytes and plasma which were compatible for the ABO blood groups resulted in clumps composed entirely of leucocytes (Figure 1). These clumps were predominantly composed of granulocytes but the presence or percentage of mononuclear cells involved was difficult to determine. These agglutination reactions were uniformly weak, difficult to reproduce and therefore difficult to study.

Positive agglutination reactions between leucocytes and plasma which were incompatible for the ABO blood groups resulted in clumps composed of a mixture of red and white cells (Figure 2c). These reactions were somewhat stronger, at times reaching a titer of 1:4. The average agglutinin, however, is not present on dilution and is variable from day to day. Table II shows the results of repeating the agglutination study on a group of these weak agglutinins daily for four days. On the other hand, the results on any one day are reproducible as shown in Table III. Erythrophagocytic indices were determined for each of these tests.

Unless otherwise noted, the following observations concern the agglutination of the mixed cell type.

TABLE III

The results of tests for leucocyte agglutination (A) and erythrophagocytosis (E), (as measured by the erythrophagocytic index) repeated five times on the same day are compared

		REPRODUCIBILITY ON SAME DAY									
PLASMA DONOR TYPE	CELL DONOR TYPE	NO. OF TESTS 1		2		3		4		5	
		A	E	A	E	A	E	A	E	A	E
O +	B +	4+	50.4	4+	39.6	4+	50.8	4+	49.6	4+	36.8
A +	B +	−	0	−	0	−	0	−	0	−	0
O +	A +	2+	26.8	2+	25.6	2+	26.4	2+	26.4	2+	*
A +	A +	−	0	−	0	−	0	−	0	−	0
O +	A +	2+	26.4	2+	19.6	2+	23.2	2+	23.3	2+	19.5

* Indicates that the test was not performed.

Physico-chemical nature of the agglutinin causing the mixed cell clumping

Some of the properties of the agglutinating substance were studied by determining its titer in several of the combinations which were incompatible for the ABO red cell types. The effect of various physico-chemical factors on these titers was then observed.

The agglutinating substance was found to be moderately thermostable resisting heating to 56° C for 30 minutes. Heating above this temperature tended to cause clotting of the plasma, however, the occasional one did not clot on heating at 65° C for 10 minutes. In these the agglutinating substance was destroyed. There was no change in the avidity of the plasma over a temperature range of 4° C to 37° C or a pH range of 6.8 to 9.0. The number of white cells between 2,500 and 20,000 cu. mm. did not influence the reaction. The ratio of plasma to white cell suspension did not affect the result if it varied between equal parts of each and 2½ of plasma to 1 part of cell suspension.

The entire group of tests for leucocyte agglutination (160 combinations of plasma and leucocyte donor) were repeated with and without albumin added and were read at one and two hours. There was no difference between incubation at one and two hours. None became positive after one hour that were not positive at that time. In 17 instances a positive reaction was present with albumin that was negative without it. Of these 17 tests smears were studied for erythrophagocytosis in 9 instances in which there was an incompatibility between the plasma and white cell donor for the ABO blood groups. In all of these instances a significant erythrophagocytic index was found in the presence of albumin and no erythrophagocytosis was produced without it.

TABLE IV

THE DISTRIBUTION OF NATURAL LEUCOCYTE AGGLUTININS*

PLASMA DONORS		CELL DONORS						
	TYPE	I O+	II O+	III A+	IV A+	V A+	VI B+	VII B+
1	O+	0	0	0	0	0	0	0
2	O+	0	0	0	0	0	+	+
3	O−	0	0	+	+	+	+	0
4	O+	0	0	0	0	0	+	0
5	O+	0	0	0	0	+	0	0
6	O+	0	0	+	0	0	0	0
7	O+	0	0	0	0	+	0	0
8	O+	0	0	+	+	+	+	0
9	O+			+	+	+		
10	O+			+	+	+	+	
11	A+	0	0	0	0	0	0	0
12	A+	0	0	0	0	0	0	0
13	A+	0	0	0	0	0	0	0
14	A+	+	0	0	0	0	0	0
15	A+	0	0	0	0	0	0	0
16	A+	0	0	0	0	0	0	0
17	A+		0	0	0	0	0	0
18	A+	+	0	0	0	0	0	0
19	A+	0	0	0	0	0	0	0
20	B+	0	0	0	0	0	0	0
21	B+	0	0	0	0	0	0	0
22	B+	0	0	+	+	+	0	0
23	AB+	0	0	0	0	0	+	0
24	AB+	0	0	+	0	0	+	0

* The results of the tests for leucocyte agglutination are shown. Agglutination is indicated by +; O denotes no agglutination; a blank space means the test was not performed.

Incidence and distribution

The plasma of 24 donors was tested against the leucocytes of 7 donors on at least two and in a few instances three occasions. In all 160 combinations were tested. Erythrophagocytic indices were determined at least once for each combination. A positive test was defined as one which showed agglutination on at least two occasions. There were 30 positive tests out of the 160 combinations (Table IV). In 84 of the 160 tests (52.5 per cent) for leucocyte agglutination the plasma samples and leucocyte donors were compatible with respect to A, B and O red cell types. In 76 of the 160 tests (47.5 per cent) the combinations were incompatible.

In 6 of the 30 tests (20 per cent) the plasma samples and leucocyte donors were compatible with respect to the ABO red cell types. In 24 of the 30 positive tests (80 per cent) the combinations were incompatible (Figure 3).

Erythrophagocytosis

When stained smears were made of the leucocytes used in the test for leucocyte agglutinins, it was noted that erythrophagocytosis was present in some.

In the 6 positive tests in which the plasma donors and leucocyte donors were compatible with respect to the A, B, and O red cell types none

showed erythrophagocytosis. Of the 24 positive tests which were incompatible 22 (92 per cent) showed a significant erythrophagocytic index. None of the 66 tests which were incompatible with respect to the A, B, and O red cell types but did not show clumping had a significant erythrophagocytic index. A significant erythrophagocytic index was not observed in the absence of agglutination (Figure 3).

Inhibition of agglutination and erythrophagocytosis by specific polysaccharide of blood group A

Nine positive leucocyte agglutination tests involving incompatible combinations due to the presence of the anti A agglutinin in the plasma were repeated in the presence of the specific polysaccharide of blood group A. All the tests were inhibited by this reagent. None of the erythrophagocytic indices was positive.

Inhibition of agglutination and erythrophagocytosis by saliva

The saliva of four out of six of the panel of cell donors so tested demonstrated the ability to inhibit leucocyte agglutination and erythrophagocytosis involving the donor's own cells. These four persons were secretors of A or B substance. The two whose saliva did not produce inhibition were non-secretors of the A or B substance. A group of 10 non-secretors of the A and B substance was studied. The saliva of these individuals was tested for its ability to inhibit an agglutination involving the donors' white cells. None of those tested inhibited the reaction.

Hemolysins and Hemagglutinins

The sera of 7 of 8 type O plasma donors which produced leucocyte agglutination and erythrophagocytosis were tested for the presence of hemolysins. In only one of those tested was an hemolysin demonstrated.

The anti A and anti B titers of the plasma donors are shown in Table V.

The C antibody of group O plasma

Five combinations of group O plasma and white cell suspensions which were positive for leucocyte agglutination and showed significant erythrophagocytosis were re-examined after absorption of the C antibody. In no instance was there less leucocyte agglutination or erythrophagocytosis after this absorption had been carried out.

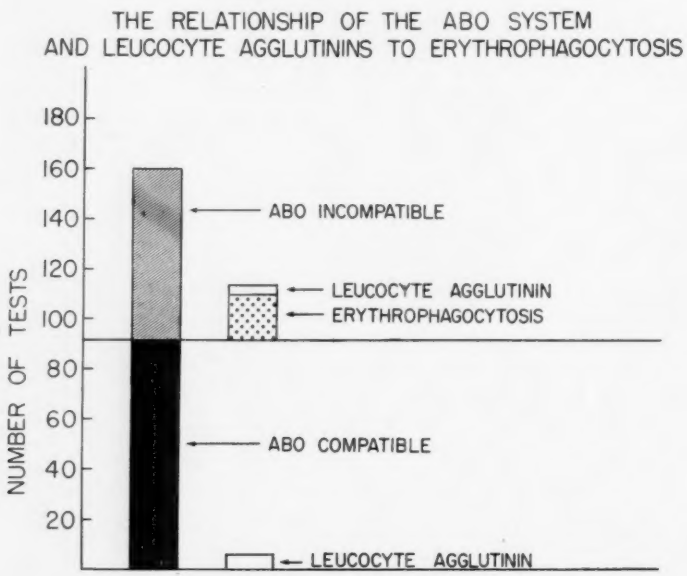

FIGURE 3

TABLE V

RELATIONSHIP OF HEMAGGLUTINATION ERYTHROPHAGOCYTOSIS AND LEUKAGGLUTINATION*

PLASMA DONORS		ANTI A	ANTI B	E.P.	LEUCOCYTE DONORS III A+	IV A+	V A+	VI B+	VII B+
8	O+	64	32	53.8	+	+	+	+	0
3	O+	256	128	20	+	+	+	+	0
22	B+	256		22.4	+	+	+	0	0
2	O+	256	128	4.8	0	0	0	+	+
5	O+	256	128	10.6	0	0	+	0	0
6	O+	128	128	4.2	+	0	0	0	0
7	O+	64	128	7.4	0	0	+	0	0
1	O+	64	64	0	0	0	0	0	0
12	A+		512	0	0	0	0	0	0
13	A+		32	0	0	0	0	0	0
14	A+		128	0	0	0	0	0	0
15	A+		256	0	0	0	0	0	0
18	A+		64	12.4	0	0	0	0	0
20	B+	64		0	0	0	0	0	0
21	B+	64		0	0	0	0	0	0

* The results of the tests for hemagglutination; erythrophagocytosis (erythrophagocytic index) and leucocyte agglutinins for some of the plasma-leucocyte combinations are shown. The numbers used to designate the plasma donors refer to the plasma donors in Table IV E.P. indicates the erythrophagocytic index.

The ability of the test plasma to cause erythrophagocytosis in the presence of incompatible red cells

This test was performed on 15 of the 24 plasmas tested for leucocyte agglutination. Eight of those investigated developed a significant erythrophagocytic index. These results are illustrated in Table V.

DISCUSSION

Various methods have been used to prepare white cell suspensions relatively free of red cells and platelets. Some have prepared them from the buffy coat after centrifugation (14). Others have used flotation techniques (15), or methods to increase sedimentation of the red cells by rouleaux formation (16). Moeschlin and Schmid (12) used heparin as an anti-coagulant and Dausset, Nenna, and Brecy (10) used defibrinated blood. In our hands centrifugation, heparin and attempts at defibrinating with glass beads all caused injury to the white cell as evidenced by a non-specific stickiness. Sequestrene appeared to be the ideal anticoagulant in preventing non-specific clumping of the white cells. Fibrinogen was discarded as a sedimenting agent for red cells because of its tendency to precipitate out of solution on incubation at 37° C. Dextran was easily available and did not seem to cause clumping.

The method described here for preparing the leucocyte suspension is simple and produces white cells which do not clump spontaneously (Figure 4). They are irregular in outline, undergo active amoeboid motion, are capable of erythrophagocytosis, in general appear to be viable. The main disadvantage of the method is that sequestrene binds the divalent cations and in this way inhibits the action of complement (17). Plasma was chosen as a diluent because saline was found to cause non-specific clumping of the white cells. It is possible that this was due to impurities in the sodium chloride. The tests were performed at pH 6.9 because others (18, 19), had found that

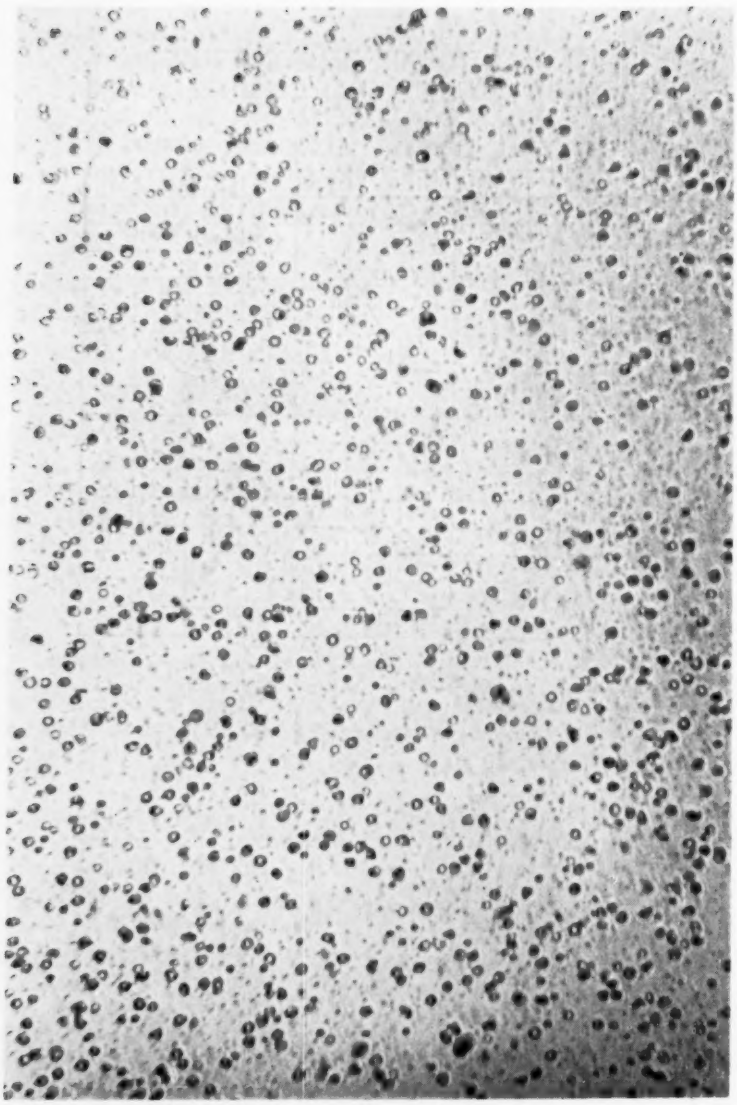

FIG. 4. PHOTOMICROGRAPH UNDER LOW POWER SHOWING THE RESULTS OF A NEGATIVE TEST FOR A LEUCOCYTE AGGLUTININ

certain hemolysins are more active at this acidity. Later in the study it was discovered that pH had little effect on the test; to maintain uniformity, however, all tests were performed at pH 6.9.

False positive clumping caused by injury to the leucocyte suspension produced a stringy irregular type of clump. The clump caused by an agglutinin was more tightly packed and symmetrical. Occasionally, however, they were difficult to differentiate morphologically. Although each leucocyte suspension was examined for signs of non-specific clumping, one could not be sure that the cells in an individual test had become injured and were clumped non-specificially. The absence of day to day reproducibility and difficulty in differentiating specific and non-specific clumping must be

considered in the interpretation of any studies of leucocyte agglutination. These have been referred to by other authors (8, 10), but have not been emphasized. Because of this, rather rigid criteria were adopted and only those which were positive on two occasions were counted. This eliminated 27 positive tests which were not present on repeat. Three of these were incompatible with respect to the A, B, and O red cell types and 24 were compatible. Undoubtedly, some leucocyte agglutinins were in this group.

The finding of erythrophagocytosis in 22 of the 24 tests which contained a mixture of red and white cells led to some speculation as to its cause. The inhibition of both the agglutination and the erythrophagocytosis by the saliva of appropriate secretors indicted the incompatibility of the plasma and cells. As the antigens Le^a and Le^b are present in saliva in addition to the antigens of the ABO blood group similar studies were carried out for inhibition by the specific polysaccharide of blood group A. This substance inhibited both leucocyte agglutination and erythrophagocytosis but no correlation was found between these two phenomena and the titer of the red cell agglutinins.

The majority of the tests which were positive for leucocyte agglutinins and erythrophagocytosis involved combinations of group O plasma and group A white cell suspensions. Unger and Wiener (20) have presented evidence that the C antibody in the plasma of a group O mother may cause erythroblastosis fetalis in a group A fetus. Absorption of the C antibody of the group O plasma, however, failed to reduce its tendency to cause agglutination of the leucocytes or erythrophagocytosis.

Bonnin and Schwartz (21) had demonstrated that an hemolysin and complement were required in addition to an hemagglutinin for erythrophagocytosis to occur due to the presence of an incompatibility for the A, B, and O red cell types. Complementary action in this system seemed doubtful due to the presence of sequestrene. Though it was not possible to test this hypothesis directly by heat inactivation as the white cell suspensions were badly damaged by heating to 56° C for 30 minutes, the fact that only one positive plasma donor out of those tested showed lysis made the usual mechanism seem even less likely.

The fact that leucocyte suspensions of the plasma donors in their own plasma caused erythrophagocytosis in certain instances when mixed with incompatible red cells ruled out the participation of a leucocyte agglutinin in the reaction as a factor. The correlation between the ability of these plasmas to cause erythrophagocytosis and to cause mixed cell clumping raised the question that the former may be related to the latter.

In order to investigate this possibility the process of mixed cell clump formation was examined under the microscope. In addition cinephotomicrographic studies were made and the following observations were noted: first, the red cells became spherocytes and clumped. Next, after about 15 minutes, the white cells became sticky and coming in contact with spherocytes became attached to them. The white cell than engulfed the red cell by surrounding it with pseudopodia. This was a complicated process and took 15 to 30 minutes. Eventually the red cell was taken into the white cell. Subsequently, two erythrophagocytes joined (Figure 2a). Cinephotomicrography revealed that this occurred in a very specific manner. When two erythrophagocytes made contact at the non-red cell pole of the leucocyte they did not stick together. The chance touching of a white cell pseudopod to the red cell pole of the erythrophagocyte, however, caused sticking of the two. It appeared that the red cell was attractive to another erythrophagocyte, even though it was inside a white cell.

Large mixed red and white cell clumps were formed by a continuation of the process of erythrophagocytes clumping together (Figures 2a, b, c).

It would appear that these mixed cell clumps were caused by a tendency to erythrophagocytosis alone, and that no white cell agglutinin was present. The reason why one plasma causes erythrophagocytosis to a greater degree than another is unexplained and this is being investigated further.

SUMMARY AND CONCLUSIONS

The plasmas of 24 normal donors were tested against the white cells of 7 normal donors. In all, 160 combinations of plasma and white cells were tested and 30 showed agglutination reactions. Two types of cell clumps were noted, the "pure" white cell clump and the mixed red and white cell clump. Six of the positive tests were of the "pure" white cell variety and these occurred in plasma white cell combinations which were compatible

for the ABO blood groups. In twenty-four of the positive tests the clumps formed were of the mixed cell type; these occurred with combinations which were incompatible. The mixed cell clump appears to be the result of a strong tendency on the part of certain plasmas to cause erythrophagocytosis rather than the action of a leucocyte agglutinin. There is no relationship between the leucocyte agglutinins and the ABO blood groups. In that naturally occurring leucocyte agglutinins do exist it would be well in studying a patient's plasma or serum for abnormal leucocyte agglutinins to test it against a panel of leucocytes which are compatible for the ABO blood groups.

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COMPARATIVE STUDIES ON THE TURNOVER OF SERUM ALBUMIN IN NORMAL HUMAN SUBJECTS [1]

By SHELDON MARGEN and HAROLD TARVER

(From the Department of Physiological Chemistry, University of California School of Medicine, Berkeley, Calif.)

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Many tracer studies involving the use of different elements have been carried out on the distribution and metabolism of plasma protein. In human experiments, the isotopic preparations used to date have been amino acids or plasma proteins labeled with N^{15} (1), S^{35} (2), C^{14} (3) and especially with I^{131} (3–6).[2] In general the techniques employed have involved measurements of the rate of loss of a label from proteins although some have considered the incorporation (6). The proteins have been initially labeled by different methods as follows: 1) Labeled amino acid has been administered either orally or parenterally to the subject, resulting in the endogenous labeling of the plasma proteins. The loss of the label, for example from N^{15} or C^{14} labeled glycine or from S^{35}-labeled cystine or methionine, has been followed *in the same subject*. 2) Endogenously labeled homologous plasma protein fractions have been transferred from a donor to a recipient. This will be referred to as the standard method (see under discussion). 3) Plasma protein fractions iodinated with I^{131} *in vitro* have been administered.

Markedly different half-lives for albumin have been reported in some of these studies (2, 3, 5), so in order to evaluate these methods, duplicate studies have been performed in normal subjects involving the use of different pairs of the methods available. One group received labeled amino acids and I^{131}-labeled albumin; a second group received both I^{131} and endogenously labeled albumin simultaneously. The results obtained exhibit marked differences depending upon the type of labeling. The significance of these differences will be dealt with in the discussion.

MATERIALS AND METHODS

1. *Preparation of S^{35}-labeled methionine and cystine:* The S^{35}-labeled methionine and cystine employed were prepared from the yeast *Torulopsis ulitis*, grown in suitable media containing S^{35}-sulfate [3] as the limiting substrate (7–9). At the end of the incubation period the yeast was collected, washed and hydrolyzed with acid. The two labeled L-amino acids were isolated using Dowex 50 columns.

In all experiments where this material was used to follow the endogenously labeled plasma protein in the same subject, the amino acids were injected intravenously. In the case of cystine, the amino acid was prepared and sterilized in the minimum of acid necessary for its dissolution, and immediately before injection it was mixed with sufficient sterilized phosphate buffer to bring the pH to 7.0. Under these conditions there was no precipitation of cystine. The methionine was dissolved as such in pyrogen-free water, sterilized by autoclaving and injected intravenously.

2. *Preparation of S^{35}-labeled plasma and albumin:* The S^{35}-labeled plasma and albumin were prepared in the following manner. A fifty-year old white male, suffering from severe pulmonary emphysema and fibrosis and chronic cardiac failure with a marked secondary polycythemia, was given a mixture of S^{35}-labeled L-cystine and L-methionine orally. Twenty-four hours later 500 ml. of blood were withdrawn. This blood was collected in 80 ml. of ACD solution as anticoagulant. The plasma was separated immediately and half was fractionated according to the Cohn technique.[4] The remaining half of the plasma was used as such. The albumin was isolated for each specimen obtained from the recipient according to the technique outlined below. This was done in order to determine whether any differences might be observable between the albumin obtained by the Cohn fractionation procedure and non-fractionated plasma. However, a paper electrophoretic study of the donor plasma protein revealed an entirely normal distribution pattern.

[1] This research was supported in part by grants from the Damon Runyon Fund for Cancer Research, from the United States Department of Health, Education and Welfare, and in part from funds administered by the Cancer Board of the University of California, San Francisco.

[2] These are only a selected group of references. Other literature citations may be obtained from these sources.

[3] We are indebted to the United States Atomic Energy Commission for supplies of S^{35} and I^{131} used in these studies.

[4] Our thanks are due to Dr. Fred Johnson of Cutter Laboratories, Berkeley, for carrying out this fractionation by Method V.

3. *Preparation of I^{131}-labeled albumin:* In the majority of the experiments reported here the albumin was iodinated according to a slight modification of the method of Pressman and Eisen (10), the I^{131} being distilled into the potassium iodide, borate buffer, protein mixture. Following this technique, between 7 and 11 per cent of the radioactive iodine distilled into the reaction flask became protein bound. The iodinated material was dialyzed against several changes of 0.01 *M* phosphate buffer at pH 6.5 until the trichloracetic acid-precipitable iodine was 97 per cent or more of the total radioactivity. The albumin solution was then passed through an ultrafine sintered glass filter and stored in the cold. The labeling generally was very light, being only approximately one atom of iodine per 36 molecules of protein.[5] One series of studies was carried out with albumin which was iodinated with ten times the usual amount of iodine. Under these circumstances there was about 0.25 to 0.35 atom of iodine per molecule of albumin. This will be referred to as lightly iodinated albumin.

In order to test whether different methods of iodination give products with altered turnover rates, a series of studies was carried out by iodinating the material according to the method of Berson, Yalow, Schreiber, and Post (5). Although they state that about 25 to 35 per cent of the I^{131} became protein bound, in our hands the yields were much poorer than this.

4. *Electrophoretic studies on iodinated albumin* [6]*:* Several lots of I^{131}-labeled albumin were studied by zone electrophoresis, performed on starch. To 25 mg. of non-iodinated albumin was added 5 mg. of the iodinated preparation and electrophoresis was performed at 4.7 volts per cm. for 16 hours in barbital buffer at pH 8.6 and with an ionic strength of 0.05.

The starch slabs were cut into sections of 5 or 10 mm. wide and each section was assayed for both radioactivity and protein content. It can be seen from Figure 1, that there were differences in the mobility between the major part of the albumin and the radioactivity. Part of the labeled material moved more rapidly than did the albumin as a whole. This is in accord with the findings of Gabrieli, Goulian, Kinersly, and Collet (11).

5. *Preparation of samples for determination of S^{35}:* The separation of the albumin fraction from the rest of the plasma proteins was attained by a two-step phosphate separation, carried out as follows: Three ml. of serum were mixed with 12 ml. of 1.75 *M* phosphate buffer, pH 6.5, and allowed to stand for a minimum of 7 hours. The precipitate of gamma globulin was removed by centrifugation. To 15 ml. of the supernatant were added 45 ml. of 2.6 *M* phosphate, pH 6.5, and the mixture allowed to stand 12 hours. This resulted in the precipitation of the remainder of the gamma globulin, the mixed alpha and beta globulins and a small fraction of the albumin. The precipitate was filtered off leaving the rest of the albumin in the supernatant. Albumin so prepared was analyzed by free boundary electrophoresis and found to be approximately 95 per cent pure, the chief contaminant being a small amount of alpha globulin.

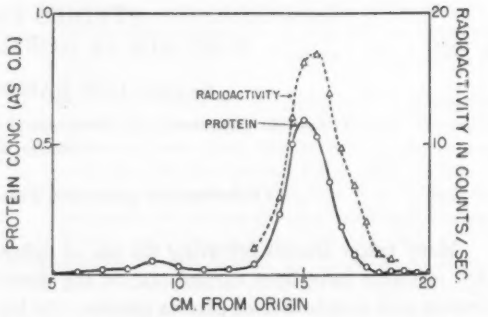

FIG. 1. STUDY OF A MIXTURE OF IODINATED AND NON-IODINATED HUMAN SERUM ALBUMIN BY THE METHOD OF ZONE ELECTROPHORESIS ON STARCH

The albumin was precipitated from the supernatant with trichloracetic acid (TCA) at a final concentration of 10 per cent and washed repeatedly with 5 per cent TCA, until the phosphate contamination became such as not to interfere with the subsequent sulfate analysis. In the case where the endogenously labeled albumin was administered, 1 ml. of total plasma protein was taken for analysis and no separation was carried out.

The protein samples labeled with S^{35} were digested with Pirie's reagent (12), brought to dryness and treated with hydrochloric acid to convert all copper salts into the soluble chloride and precipitated with benzidine dihydrochloride. The benzidine sulfate was then filtered onto Whatman No. 1 filter paper and counted directly on this paper. During the process of digestion any iodide (I^{131}) from the double labeling was lost, since there was no detectable I^{131} in any of the samples after this type of digestion.

After the samples were counted the benzidine sulfate was titrated with standard sodium hydroxide (13).

6. *Preparation of samples for I^{131} determination:* For I^{131} determination 1 or 2 ml. of plasma or serum, in an aluminum dish rimmed with silicone stopcock grease, were thoroughly mixed with 1 ml. of a 1 per cent TCA solution to which a small amount of detergent was added. The solutions were allowed to dry slowly over a hot plate. Under these conditions a smooth uniform layer of protein was easily obtained.

7. *Determination of radioactivities:* Radioactivities were determined in either a thin mica end window Geiger-

[5] The ordinary procedure of iodination involved the use of 100 mg. of albumin iodinated with the iodine from 0.02 ml. 0.02 *M* iodide: *i.e.*, micromoles of albumin (M. W. 69,000) = 1.45, microatoms of iodine used = 0.4; so on the basis of a 10 per cent yield, the actual amount of iodine used was 0.04 microatoms, or approximately 1 atom iodine per 36 moles of albumin. The ratio may be somewhat higher than this if the radioiodine came into equilibrium with the iodide in the system (1:24).

[6] Our best thanks are due to George Bagatella for his assistance and advice in carrying out the experiments involving electrophoresis.

Mueller tube, in a gas flow tube, or in the tube with gas flow and a thin pliofilm window described below. Since most of the samples that were counted were double labeled (both $I^{131}$and S^{35}) it was necessary in this counting procedure to screen out the S^{35}. This was done by the use of a mica shield of sufficient thickness to absorb over 97 per cent of the soft-β radiation from the S^{35}. The possible contribution of the S^{35} to the counting rate was less than 1 per cent of the iodine.

In earlier work the S^{35} samples were counted on a windowless flow counter. However, this method was discarded in favor of a modified flow counter which consisted of a simple bell-type Geiger tube with the opening about 2½ inches in diameter. Over this was stretched a thin pliofilm window about 0.3 mg. per sq. cm. thickness. Because of the permeability of this film to gas, it was necessary to pass the counting gas, helium saturated with ethanol at 0°, through the tube during counting. This tube proved to be very stable, although it cut down counting efficiency by about 50 per cent over the use of a windowless counter. However, the stability of the tube more than made up for the loss of efficiency.

Since all protein samples were prepared in a similar way no correction was necessary for self absorption, but all benzidine sulfate samples were corrected for both physical decay and self-absorption. Counting was carried out long enough to achieve a statistical accuracy ± 5 per cent.

8. *Subjects:* All subjects studied were adult males and with one exception ranged in age from 20 to 30. The remaining subject was 45. They were all ambulatory and at their customary work. The diet was not controlled, but appeared to be adequate. The blood was collected and either allowed to clot or placed into a Wintrobe ammonium-potassium oxalate mixture.

The subjects who received I^{131}-labeled material were divided into two groups; the first group did not receive any iodide to block thyroid uptake of radioiodine; the larger group received ten drops of saturated solution of potassium iodide, twice daily, throughout the duration of the experiment.

9. *Administration of radioactive material:* The I^{131}-labeled albumin was administered intravenously in a dose of 5 to 10 mg. containing approximately 10 to 15 microcuries. Blood samples were taken within 15 min. in all instances, in order to establish the circulating blood volume. Subsequently several specimens were taken during the first day followed by specimens every twenty-four hours for the first week and three times weekly thereafter as long as counting was feasible.

When the S^{35}-labeled cystine or methionine was administered the dose again was given intravenously, amounting to between 20 and 30 microcuries in 5 to 10 mg. of material. Blood samples were taken either twice on the first day or more frequently. Beginning twenty-four hours after the dose, samples were then collected daily for the first week and either two or three times weekly for the next two weeks thereafter. Subsequently samples were taken at varying intervals up to 60 days.

In cases where the S^{35}-labeled plasma or albumin were administered 1.25 to 2 gm. of material containing 5 to 10 microcuries of S^{35} were injected intravenously.

I^{131} and S^{35} were determined on the same samples of blood.

10. *Calculation of results:*

(a) *The plasma volume and albumin in the vascular circulation:* This was calculated directly from the dilution found in the first blood sample; *e.g.*, from the iodine curve of Wil (Figure 4), the total dose of 8.02×10^6 c.p.m. was diluted to 3.10×10^3 c.p.m. per ml. Hence, plasma vol. $= \frac{8.02 \times 10^6}{3.10 \times 10^3} = 2{,}590$ ml. and the rapidly circulating albumin = 2,590 × 0.053 = 137 gm. where the per cent albumin in the phosphate fractionated serum was found to be 5.3 by micro Kjeldahl analysis. Similar values for plasma volume and circulating albumin were found by the standard method.

(b) *Total albumin:* This was also calculated from the dilution using the extrapolation of the metabolic portion of the exponential curve, *e.g.*, in the case of Wil, the second linear portion of the curve being employed. Hence, total albumin $= 137 \times \frac{3.10 \times 10^3}{5.6 \times 10^2} = 760$ gm. This value does not agree with that given in Table IV from the albumin-S^{35} data (426 gm.) for reasons given in the discussion. From this value of 760 gm. the ratio of total albumin to the albumin in the blood vascular circulation is 5.5.

(c) *Rate of synthesis:* The calculation of the rate of protein synthesis rests on the assumption that all the radioactivity lost from the protein is lost due to net destruction, neither the amino acid nor its label exchanging out of the protein.

The half-life of the protein is determined graphically, in the case of Wil, being 15 days. Then,

Synthetic rate as

$$\text{per cent per day} = \frac{100 \ln 2}{t_{\frac{1}{2}}} = \frac{69.3}{15} = 4.6 \text{ per cent}$$

or,

Synthetic rate as

$$\text{gm. per day} = \frac{4.6}{100} \times 760 = 35 \text{ gm.}$$

This figure should be contrasted with the figure of 12.3 gm. per day given in Table IV. The higher value is in error due to the operation of several factors which are dealt with in the latter part of the discussion.

RESULTS

Experiments with I^{131}-labeled albumin

Figure 2 shows the results of plotting the average values obtained with three normal subjects given I^{131}-albumin. Further details are given in Table I. The curve is essentially the same as those obtained by others (2, 4, 5), and can be resolved into at least two and possibly three com-

TABLE I

Short term studies in which normal human subjects were given human serum albumin very lightly labeled with I^{131}

Subject	Weight *kg.*	Period followed *days*	Albumin *gm. per cent*	Circulating albumin *gm.*	Total albumin *gm.*	Ratio total to circulating albumin	Half-life *days*	Albumin synthesized *gm. per day*	Albumin synthesized *gm. per kg. per day*
Bon.	71	9	5.22	150	406	2.7	11.0	25.6	0.360
Cre.	68	9	4.26	120	312	2.6	9.0	24.0	0.350
Ele.	74	12	4.98	147	456	3.1	13.0	24.6	0.330
Koe.	77	8	5.05	146	380	2.6	10.0	26.2	0.392
Mas	62	13	4.03	96	211	2.2	9.3	15.8	0.253
Spa	66	12	4.66	123	283	2.3	9.0	22.0	0.330
Tar.	70	9	4.94	130	429	3.3	9.4	31.8	0.454
							Mean and standard deviation		0.346+0.054

ponents. The first component is assumed to indicate intravascular, extravascular mixing. The second component, with the half-life of about forty hours, may represent the intracellular, extracellular equilibrium of the I^{131}-albumin, whereas the third portion of the curve represents the metabolic destruction of the I^{131}-albumin. From the extrapolation of the third portion of the curve it is possible to calculate the total albumin as previously indicated.

In one experiment during the preparation of the I^{131}-labeled albumin, the material was inadvertently denatured. Although the denaturation was not sufficient to noticeably change its solubility, the effect upon the metabolic handling of the material was very pronounced as shown in Figure 3. It is seen that the denatured albumin was very rapidly removed from the circulation so that in twenty-four hours there was virtually none demonstrable.

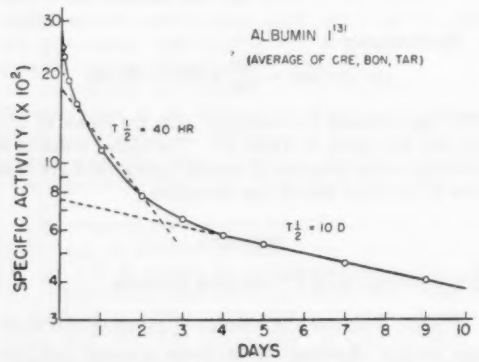

FIG. 2. SEMI-LOGARITHMIC PLOT SHOWING THE RELATIONSHIP BETWEEN THE SPECIFIC ACTIVITY OF PLASMA PROTEIN AND TIME FOLLOWING THE INJECTION OF VERY LIGHTLY IODINATED HUMAN SERUM ALBUMIN IN THREE NORMAL HUMAN SUBJECTS

In Figure 4 and Table II are shown the results obtained when samples of albumin iodinated with two different amounts of the halogen were used in comparable studies. Curve with triangle symbols represents the values obtained with the I^{131}-albumin with about one atom of iodine per 36

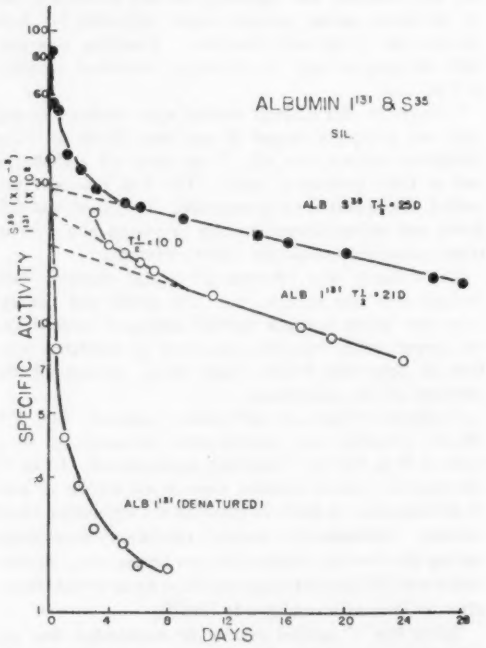

FIG. 3. SEMI-LOGARITHMIC PLOT SHOWING THE RELATIONSHIP BETWEEN THE SPECIFIC ACTIVITY OF PLASMA PROTEIN AND TIME; (a) FOLLOWING THE INJECTION OF ENDOGENOUSLY LABELED ALBUMIN, (b) FOLLOWING THE INJECTION OF VERY LIGHTLY IODINATED HUMAN SERUM ALBUMIN AND, (c) FOLLOWING THE INJECTION OF DENATURED VERY LIGHTLY IODINATED ALBUMIN

All experiments carried out on the same subject.

TABLE II

Long term studies in which normal human subjects were given human serum albumin lightly or very lightly labeled with I^{131}

Subject*	Period followed days	Values derived from the fast component of the turnover data: Circulating albumin gm.	Total albumin gm.	Ratio total to circulating albumin	Half-life days	Albumin synthesized: gm. per day	Albumin synthesized: gm. per kg. per day	Values derived from the slow component of the turnover data: Total albumin gm.	Ratio total to circulating albumin	Half-life days	Albumin synthesized: gm. per day	Albumin synthesized: gm. per kg. per day
Cos.	22	173	450	2.6	8.	28.4	0.383	690	4.0	17	38.9	0.525
Gal.	27	154†	324	2.1	9.8	19.6	0.288	482	3.1	17	22.9	0.337
Mar.	22	125	275	2.2	8.4	19.6	0.258	425	3.4	15	22.5	0.295
Rei	19	145	464	3.2	8.5	34.0	0.500	740	5.1	15	37.9	0.555
Rus	24	168†	385	2.3	8.	21.4	0.268	770	4.6	25	33.4	0.416
Sil	24	172†	388	2.2	10.	17.5	0.227	533	3.1	21	24.4	0.317
Ste	27	120†	305	2.5	8.2	17.9	0.271	466	3.9	18	25.8	0.392
Wil	22	137	410	3.0	8.5	34.6	0.525	755	5.5	15	33.3	0.504

* The weights and per cent albumin in the circulation of the subjects used in these studies are to be found in the other tables.

† Values obtained from S^{35}-labeled protein experiments.

moles of albumin (very lightly iodinated). The other two curves with open circles show the values obtained with albumin iodinated 10 times as heavily (lightly iodinated). This, therefore, would represent about 0.3 atoms of iodine per mole of albumin. As can be seen in the two curves for the first ten-day period there is no noteworthy difference between the results with the albumin iodinated in either of these ways. However, it should be noted that the so-called heavily iodinated material used in these studies is actually lightly iodinated by the generally accepted standards.

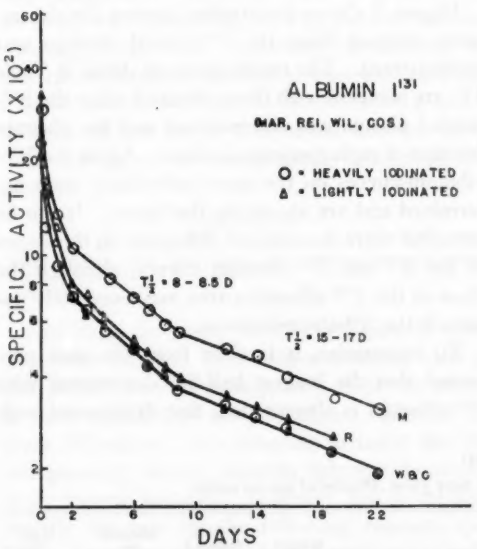

FIG. 4. SEMI-LOGARITHMIC PLOT SHOWING THE RELATIONSHIP BETWEEN SPECIFIC ACTIVITY OF PLASMA PROTEIN AND TIME IN NORMAL SUBJECTS GIVEN HEAVILY IODINATED AND LIGHTLY IODINATED HUMAN SERUM ALBUMIN (LIGHTLY AND VERY LIGHTLY IODINATED PREPARATIONS BY GENERALLY ACCEPTED STANDARDS) (SEE TEXT)

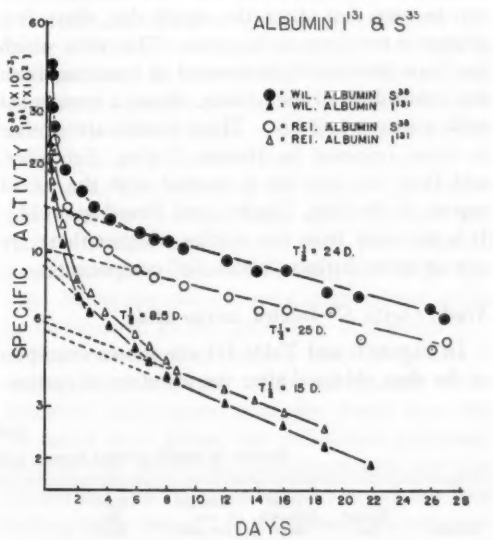
ALBUMIN I131 & S35
● = WIL. ALBUMIN S35
▲ = WIL. ALBUMIN I131
○ = REI. ALBUMIN S35
Δ = REI. ALBUMIN I131
SPECIFIC ACTIVITY S35 (X 10⁻³) / I131 (X 10²)
T½ = 24 D.
T½ = 8.5 D.
T½ = 25 D.
T½ = 15 D.
DAYS

FIG. 5. SEMI-LOGARITHMIC PLOT SHOWING THE RELATIONSHIP BETWEEN SPECIFIC ACTIVITY OF PLASMA PROTEIN AND TIME IN TWO NORMAL SUBJECTS GIVEN BOTH VERY LIGHTLY IODINATED HUMAN SERUM ALBUMIN AND ENDOGENOUSLY LABELED ALBUMIN FOR PURPOSES OF DIRECT COMPARISON

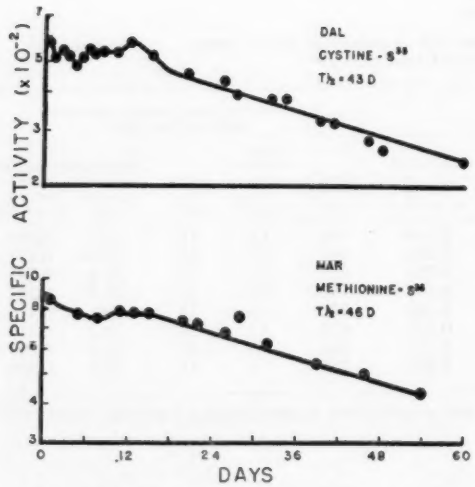

FIG. 6. SEMI-LOGARITHMIC PLOTS SHOWING THE RELATIONSHIP BETWEEN THE SPECIFIC ACTIVITY OF SERUM ALBUMIN AND TIME IN REPRESENTATIVE NORMAL SUBJECTS FOLLOWING THE ADMINISTRATION OF S^{35}-CYSTINE OR S^{35}-METHIONINE

In Figures 3, 4 and 5 are shown the results from studies in which the activity from iodinated albumin was followed for more than 10 days. It can be seen that about the eighth day, there is a change in the slope of the curve. The curve which has been previously interpreted as resulting from the metabolism of the protein, shows a component with a longer half-life. These results are similar to those reported by Berson, Yalow, Schreiber, and Post (5), but are in conflict with the recent report of Sterling, Lipsky, and Freedman (14). It is not clear from our studies whether there are two or more distinct "metabolic" components.

Studies with S^{35}-labeled amino acids

In Figure 6 and Table III are shown examples of the data obtained after the injection of cystine-S^{35} or methionine-S^{35}. It may be noted from both of these curves that the specific activity of the albumin shows no significant change for a period of about ten to fourteen days, after which there is a very slow fall. From these experiments, the biological half-life of the albumin appears to be in the neighborhood of thirty-five to fifty days or more. These results are in substantial agreement with those reported in the literature (2, 15, 16).

S^{35}-labeled plasma and albumin experiments

In Table IV are presented the data from the experiments in which S^{35}-labeled "ACD" plasma was administered intravenously to a recipient and the plasma albumin isolated. One typical curve is shown in Figure 3. The albumin-I^{131} behavior was determined simultaneously, the data being given in Table II. The latter data lie on a typical two or three phase exponential curve as was noted previously with the I^{131} albumin. However, the data do not show the typical exponential behavior until approximately the sixth to the eighth day. Subsequently, the metabolic half-life in these experiments varied from twenty-five to thirty days.

Figure 5 shows two typical curves for the results obtained when the S^{35}-labeled albumin was administered. The results given in detail in Table IV, are identical with those obtained when the S^{35}-labeled plasma was administered and the albumin fraction of each specimen isolated. Again the I^{131}-albumin-curves on the same individuals were determined and are shown on the figure. It can be seen that there is a marked difference in the slopes of the S^{35} and I^{131}-albumin curves, although the slope of the I^{131}-albumin-curve may eventually approach the S^{35}-albumin-curve.

To summarize, it is clear from the data presented that the longest half-life determined with I^{131}-albumin is shorter than that determined with

TABLE III

Studies in which normal human subjects were given S^{35}-labeled amino acids

Subject	Weight *kg.*	Period followed *days*	Albumin *gm. per cent*	Half-life *days*	Subject	Weight *kg.*	Period followed *days*	Albumin *gm. per cent*	Half-life *days*
Fin	89	35	4.64	>60	Pea*	69	29	5.28	43
Dal*	71	60	3.99	43	Sch	63.5	34	4.66	>60
Mar	76	54	4.80	46	Spa	66	28	4.66	35
Oco	76	33	4.81	>60	Wil	66	51	5.30	51
Oso	74	40	4.39	44					

* These subjects received S^{35}-cystine. All other subjects received S^{35}-methionine.

TABLE IV

Studies in which normal human subjects were given protein endogenously labeled with S^{35}

Subject	Weight kg.	Period followed days	Albumin gm. per cent	Circulating albumin gm.	Total albumin gm.	Ratio total to circulating albumin	Half-life days	Albumin synthesized gm. per day	Albumin synthesized gm. per kg. per day
Cos	74	27	5.24	173	485	2.8	26	12.9	0.174
Gal	68	49	5.22	154	462	3.0	30	10.6	0.156
Hor	86	51	5.27	182	546	3.0	27	14.0	0.163
Rei*	68	27	5.10	145	435	3.0	25	12.1	0.178
Rus	80	40	5.35	168	562	3.4	27	14.6	0.183
Sil	77	50	5.37	172	524	3.05	25	14.6	0.189
Ste	66	41	4.91	120	518	4.3	28	12.8	0.214
Wil*	66	39	5.30	137	426	3.1	24	12.3	0.187
							Mean and standard deviation		0.180±0.022

* These subjects received endogenously labeled S^{35}-albumin. All others received whole plasma in which all the proteins were endogenously labeled.

endogenously labeled S^{35}-albumin. Furthermore, if one considers only the first ten to twelve days of the I^{131}-labeled albumin experiments, the differences between the experimental results and those obtained with the endogenously labeled material are very marked. The rate of metabolism of the I^{131}-labeled albumin during this phase is 2½ to 3 times more rapid than the rate observed with the endogenously labeled protein. It is only after about the eighth day that there appears to be some slowing of the rate. However, even then the slowest component observed in most instances had a half-life of about fifteen to seventeen days, which still was almost half as long as the average metabolic half-life of the endogenously labeled plasma protein.

DISCUSSION

Concerning the standard for turnover measurements

Assuming the reality of the difference in turnover rates obtained by the three methods used in this investigation, it is necessary to try to arrive at some satisfactory interpretation to account for these differences. It is tempting to assert that the endogenously labeled albumin behaves identically to the natural material. However, this is not necessarily the case for the following reasons: (a) The material was obtained from a pathological subject; hence, it may be an aberrant form of protein (albumin). There is no proof that the albumins from all subjects, normal or pathological, are the same, in fact some investigators have reported significant heterogeneity (17), at least one different species of albumin (mercaptalbumin) having actually been isolated (18); (b) in processing or in the ageing the material may have undergone some change or it may contain some impurity, radioactive or proteinaceous; (c) the size of the dose may have resulted in some change in the rate of metabolism of the material.

However, it is improbable that any of these factors are of real importance. First, with regard to the source—the turnover rates of internally labeled albumin obtained by other investigators (2) using different donors are essentially the same as those obtained in these studies. Since the subjects used in the studies mentioned were normal, it is evident that the albumins were metabolically similar, and in all probability both were normal.

Second, with regard to the processing—similar results were obtained with unfractionated material. Hence, any change must have resulted from the separation of cells or was due to the ageing. No ageing changes were observed nor are to be anticipated, so this possibility is discounted.

Furthermore, it is unlikely that the protein is contaminated with any significant amount of extraneous radioactivity since the donor was bled 28 hours after giving the radioactive precursor. During this period the precursors and any contaminating material must have fallen to a low level in the plasma sample. If the albumin was contaminated with a small per cent of other labeled protein, since this is also undergoing turnover, it should not have made any great difference in the albumin picture, although others have entertained this possibility (15).

Third, with respect to the dose—if the size of the dose did affect the metabolism, the only logical change to anticipate would be that of a higher rate, *i.e.*, a more rapid catabolism till such time as the extra protein was removed, followed by a lower rate later in the experiment. No such effects were observed with the albumin; the logarithmic plots remained linear, except for the initial days. Differences might have been concealed in the non-linear period, but did not exist elsewhere. In view of these considerations the endogenously labeled albumin will be taken as the standard of reference, and the method involving the administration of such protein will be referred to as such.

The turnover of proteins after endogenous labeling with S^{35}-labeled amino acids

If we turn to the differences between the standard turnovers and those found by the endogenous (cystine or methionine) labeling method, the extension of the half-life must be explained. The only reasonable explanatory basis exists in the re-entry (reutilization) phenomenon. This aspect of the use of isotopes in studies of plasma protein metabolism has been emphasized by McFarlane (18) and Niklas and Poliwoda (16).

In employing the kinetic approach which is used by all recent workers (1) in this field, the tacit assumption is made that after the protein is once labeled no more labeled material becomes incorporated. It is assumed there is no reutilization of either labeled amino acid or any labeled protein fragment of a larger size; the specific activity of any precursor of the protein is, in effect, taken to be zero. Unfortunately, there is no data extant, to our knowledge, relative to the specific activity of the free cystine and methionine, during the period of turnover measurements in human subjects. However, the specific activity of the glycine pool at intervals following the administration of glycine to rats and human subjects has been measured by various workers (20–23). A rapid fall in the activity was observed. Large dilutions of the radioactive material were found. This speaks for the existence of either a large glycine pool, or for the dilution of the endogenous glycine by the dietary glycine.

However, even though dilutions observed are large, according to the work of Berlin, Hewitt, and Lotz (23), they are not sufficient to negate effects in the measurements of the life of the heme in hemoglobin in human subjects following the labeling of the red blood cells by feeding glycine-2-C^{14}. In these experiments estimates of the specific activity of the free glycine available for synthesis were made by determining the activity in urinary hippurate at intervals during the experiment. When these data were applied as a correction to that of the heme there was a significant change in the values.

In spite of this, when Masouredis and Beeckmans (3) measured half-lives of albumin in human subjects by feeding glycine-2-C^{14} values of 28 and 39 days were found in two subjects (one with polycythemia and the other with rheumatic heart disease), values about twice as long as with I^{131}-albumin in the same subjects. Presumably if these values had been corrected for the glycine which was reincorporated the half-lives would have been shorter and thus nearer to the average observed in the present studies, 26 days. Thus the re-entry phenomenon assumes importance in the method of endogenous labeling.

At this point it should be noted that according to other investigators (2) it is possible there is significant re-entry even when the endogenously labeled protein only is injected because tissue protein must become labeled to some extent even under these conditions. This is evident from the animal work of Abdou and Tarver (24, 25).

At any rate it is clear that from a comparison of the results with glycine on the one hand and those with cystine and methionine on the other that the re-entry phenomenon with the non-essential amino acids with a high rate of endogenous formation must be of lesser importance than with the essential sulfur-containing amino acids, the sulfur of which may not be in equilibrium with dietary sulfur. The amino acid pool may be inhomogeneous as is the pool for other amino acids (20) and the dietary amino acid may not be mixed completely with labeled endogenous amino acid arising from albumin breakdown (15).

The method employed to assess the specific activity of the precursor pool in many glycine studies (20–23), suggests that similar methods be used in the cystine studies. It should be possible to assess the variable by employing brombenzene, or some similar aromatic compound, to transfer

cystine into the form of mercapturic acid in the urine in sufficient amount to permit the determination of its specific activity.

The behavior of iodinated protein

It remains to consider the difference in behavior between the endogenous protein and the iodinated protein. The main possibilities in this case may be summarized as follows: 1) The albumin is modified during the process of iodination, and is rendered metabolically more labile due to partial denaturation, or oxidation of sulfhydryl groups or other reactions; 2) the metabolic system can distinguish between the iodinated protein and normal protein; 3) the iodinated protein consists of molecules which are iodinated to different degrees, and hence possess different degrees of metabolic lability; 4) a special type of albumin molecule has been iodinated preferentially; 5) iodine may be lost from or exchanged out of the protein; 6) iodine released from protein may be reincorporated into protein appearing in the plasma.

1) With regard to the first of these possibilities, the amount of iodine used to iodinate the protein was very small in most of the studies presented; namely, 0.03 to 0.3 atom per mole (69,000) of albumin. When such amounts of iodine are used for iodination, it is impossible to detect any significant decrease in the number of thiol groups in the protein by the method of Boyer and Segal (26).[7] In addition, there is no visual evidence of denaturation. Moreover, when the amount of iodine used was increased ten fold, the product yielded approximately the same half-life as the very lightly iodinated material. There is, therefore, no evidence in our data to indicate that the protein suffered any modification beyond iodination. However, for more heavily iodinated proteins differences in metabolism have been detected (5).

The fact that in some of our data and in that published by others (2, 5) there is no stable half-life with the iodinated protein, may be explicable on the basis of partial modification of some of the protein. It may equally well be explained as indicated under 3) or 4).

2) Whether the metabolic system can distinguish between the weakly iodinated and normal protein is an open question, the answer to which is probably in the affirmative. No doubt, the iodination of the protein influences the pK's of the various tyrosine residues, and so may very well modify their ability to combine with proteolytic enzymes, and hence may affect the lability of the protein, or may affect the ease with which the protein is denatured.

3) With respect to the possibility that different residues in the protein or proteins are iodinated to different degrees, it is probable that such differences occur when different amounts of iodine and different methods of iodination are employed. It may well be that the proportions existing between mono- and di-iodinated tyrosine, histidine (10, 27) and perhaps other iodinated species vary between different albumin molecules in any one batch as well as between different types of iodinated preparations. The extent to which such differences may exist appears to be in need of investigation, since the different species may be metabolized at different rates.

4) Various workers have reported on special types of albumins. The most noteworthy of these is the mercaptalbumin first described by Hughes (17, 18). But clearly, it is impossible to assess differences in lability of such preparations without, at least, their prior identification and isolation.

5) The possibility that iodine may be exchanged out of the protein needs to be considered in view of the work of Miller, Anderson, Madison, and Salley (28), who showed that under the proper conditions iodine may be exchanged into or out of diiodotyrosine. Were such a reaction to proceed with the albumin-bound iodine *in vivo*, the effect would be to give a spuriously short life. It is equally possible that iodine might be lost from the protein by reduction or some other reaction. Since we have found that the half-life of I^{131}-albumin is not altered by the feeding of extra iodide, exchange reactions may not occur *in vivo* to any significant extent.

6) It is possible that in some of our experiments, iodine from I^{131}-albumin was released and reincorporated into other proteins appearing in the plasma. However, such a phenomenon could only

[7] For these tests 0.3 micromole of the albumin preparation were dissolved in 3 ml. of acetate buffer of pH 4.6 and ionic strength 0.2. After adding 3 ml. of the *p*-chloromercuribenzoate reagent there was no difference detectable in the absorbancy readings at 255 millimicrons (Beckman D.U.), between iodinated and untreated samples.

have led to the finding of a spuriously long half-life for the protein. Since we are concerned with explaining short half-lives, this possibility will not be considered further.

The size of the metabolic pool

In the preceding paragraphs consideration has been given to the importance of the specific activity and the size of the precursor pool, in arriving at an interpretation of the turnover data. It is of equal, if not greater, importance to consider the size and specific activity of the protein pool if any significant interpretations are to be made from turnover data obtained from human subjects with various diseased states in which the rate of metabolism of protein may or may not be deranged. Unfortunately, this aspect of the problem was not considered in the report of previous investigators (13), nor has it been given sufficient weight in many other more recent communications. In this connection it is clear that the endogenously labeled plasma protein method possesses great advantages over the feeding of amino acids insofar as the actual assessment of the condition of the individual is concerned with respect to the rate of protein metabolism. This is because with the endogenously labeled material transferred from donors it is possible to make an assessment of the size of the protein pool. ***Without both a knowledge of the pool size and the half-life of the protein it is virtually impossible to arrive at any significant conclusions with regard to the actual rate of metabolism of the protein*** (29). For any given half-life of a protein in the organism, the actual rate of replacement, of synthesis and degradation, may have any value depending on whether the protein pool is normal, large or small in size.

It is for this reason that the results with the iodinated protein give a particularly erroneous value for rates of replacement. Since the slope of the metabolic portion of the half-life curve is overestimated, the intercept of the extrapolation of this curve to zero time leads to high values for the zero time specific activity of the protein. Hence, the size of the pool is underestimated. However, a second source of error exists in that the whole iodinated protein curve is generally displaced downward on the vertical axis. Thus, when the turnover rate is calculated from the quotient of pool size and half-life the net result may be in serious error, as illustrated by the calculations in the case of Wil (see under Materials and Methods). In this instance the turnover with the endogenously labeled plasma protein was found to be 12 gm. per day, whereas the iodinated protein gave a value of 35 gm. per day, nearly a three fold difference.

Where ascites are present it is possible to treat the data as if two protein pools exist, one in the body as a whole and one in the ascites, where the two pools have different rates of turnover (30, 31). To some extent the same situation must exist in the body as a whole because of the lack of homogeneity in the protein pool, the lymph albumin no doubt not having the same specific activity as the plasma albumin, for instance.

No elaborate statistical treatment has been applied to the data, since this does not appear to be justified at the present time due both to the paucity of the material, and to the fact that a complete assessment of the sources of error in the experimental work has not been made. Therefore, the average values given in the tables should be treated with caution until such time as further material becomes available. This is particularly true of the estimates of rates of synthesis which, due to the methods involved in their calculation, are prone to subjective errors.

SUMMARY

An attempt has been made to assess, in normal subjects, three methods for the measurement of albumin turnover. The method taken as the *standard of reference* involves the administration of sulfur labeled amino acid to a donor subject and the investigation of the behavior of the labeled protein from the donor in the recipient. This method is compared in the same subjects with methods involving either the injection of iodinated albumin or of S^{35}-methionine or cystine.

In agreement with other workers, the longest half-lives are found with the last method using either S^{35}-methionine or cystine and the shortest half-lives are found with iodinated albumin. However, with the iodinated protein no stable half-life is apparent. There appears to be a continuous slowing in the metabolism of the material so that the half-life of albumin measured by the standard

method involving the use of endogenously labeled protein from a donor was found to be 26.5 days in 8 normal adult male subjects.

It is pointed out that the measurement of pool size is of extreme importance in such studies as these. Only when such measurements are made is it possible to calculate replacement rates (turnover), *i.e.*, actual rates of synthesis and degradation. Erroneous ideas concerning such rates are obtained from measurements with the iodinated albumin. With the standard method the rates in the subjects mentioned are 13 gm. per day from a pool of approximately 500 gm.

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PHYSICAL ACTIVITY AND THE DIET IN POPULATIONS DIFFERING IN SERUM CHOLESTEROL [1]

By ANCEL KEYS, JOSEPH T. ANDERSON, MARIO ARESU,[2] GUNNAR BIÖRCK,[3] JOHN F. BROCK,[4] B. BRONTE-STEWART, FLAMINIO FIDANZA,[5] MARGARET HANEY KEYS, HAQVIN MALMROS,[3] ARRIGO POPPI,[6] TEODORO POSTELI,[6] BENGT SWAHN,[3] AND ALFONSO DEL VECCHIO [7]

(*From the Laboratory of Physiological Hygiene, University of Minnesota, Minneapolis, Minn.*)

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Previously we have reported that samples of men from various populations differ in the average concentration of cholesterol in the blood serum so that, in general, the cholesterol level tends to be directly related to the proportion of the total calories provided by fats in the habitual diet of the population (1–6). Differences in the cholesterol content of these diets cannot account for the differences in the serum (7).

These observations are confirmed by other recent studies (8), but the question has been asked as to whether differences in habitual physical activity could not account for the findings. The suggestion has been made that "a large muscle mass or a large energy expenditure is the effective agent and that this, not the diet, is the important element in preventing hyperlipemia" (8, p. 1010).

In some areas it is easy to show that the more prosperous men, who are usually less active physically than the rest of the local population, tend to have higher cholesterol values than men representing the rest of the community. We found this to be true in Madrid, Spain (4), in Naples, Italy (9), and in Cape Town, South Africa (6). Superficial consideration of these data might suggest that the observed cholesterol differences are causally related to physical activity since men in the more prosperous groups often tend to be relatively inactive physically.

It is now well established experimentally that changes in the proportion of calories provided by fats in the diet, without change in total calories or in physical activity, produce changes in the serum cholesterol concentrations in man (10–17) rats (18) and rabbits (19). Such evidence does not exclude the possibility that physical activity may also have an effect on the serum cholesterol level and, in fact, a slight effect is indicated in acute experiments on man (20, 21).

But the relative unimportance of differences in physical activity was indicated from studies in Naples, Italy, and in Malmö, Sweden. In Naples and Malmö, clerical workers, members of the city fire departments and heavy manual workers were studied in parallel from which it appeared that the dietary and not the physical activity characteristic of the group is the major factor in regard to serum cholesterol (5, 22). Much new evidence from Bologna, Italy, Sardinia, Minnesota and South Africa, is now at hand to allow a more precise examination of the question. The present paper summarizes these data and comparisons in regard to the factor of physical activity.

METHODS AND MATERIALS

In all cases the population samples comprised men presumed to be reasonably representative of their class in the community who were gainfully employed at their regular and habitual occupation and who were judged to be clinically healthy on the basis of a brief interview, a

[1] The collection and analysis of the data cited here was made possible by grants of funds and other assistance from the American Heart Association, New York, the Minnesota Heart Association, St. Paul, the National Dairy Council, Chicago, The American Dairy Association, Chicago, the Schweppe Foundation, Chicago, the South African Council on Scientific and Industrial Research, Johannesburg, Mr. David Winton and Associates, Minneapolis, and interested organizations at Lund and at Malmö, Sweden.

[2] Clinica Medica, University of Cagliari, Sardinia, Italy.

[3] Departments of Medicine, University of Lund, Lund and Malmö, Sweden.

[4] Department of Medicine, University of Cape Town, Union of South Africa.

[5] Institute of Physiology, University of Naples, Italy.

[6] Clinica Medica, University of Bologna, Italy.

[7] Department of Pharmacology, University of Milan, Italy.

physical examination and, in most of these series, a 9- to 12-lead electrocardiogram.

In the present study there was no effort to obtain stratified samples of men representing all modes of life, income and other characteristics in their community. The purpose was to study samples of men conforming to broad categories of occupational physical activity, who would be comparable in age and clinical "health," whose diets could be at least roughly specified in regard to fat content, and whose general manner of life, including their diets, did not depart grossly from that of the other men of similar category in their own community.

In each case arrangements for the study were first made with the employers, labor union officials and influential leaders in the job category so that the major selection had to do with the organization and not with the individuals. No organizations with unorthodox methods of employment or selection of men were involved. Uninvited "volunteers" were discouraged from seeking appointments as were men of unusual habits, diet or health notions or past history. Pressures of various kinds were employed to assure that the men actually studied represented a large and unbiased fraction of all of the men in each job category and age group in the various organizations selected for study.

The actual physical activity on the job, some personal habits such as smoking and the use of alcohol, and adherence to or departure from the general local dietary pattern were ascertained by interviewing each man at the time of examination. Men found to show signs or histories of clinical disorder or whose diets were discovered to be atypical of the local group were discarded; fortunately these cases were rare. A few men whose physical activity did not conform to the expectation on the basis of job title were re-classified.

Except in rare instances, blood samples were drawn in the morning after a short rest in the supine position and before taking the first heavy meal of the day. The relative unimportance of achieving strictly basal conditions for serum cholesterol studies has been reported elsewhere (20).

Serum total cholesterol was measured, usually in duplicate, by a slight modification (23) of the method of Abell, Levy, Brodie, and Kendall (24). Identical cholesterol standards, prepared and checked in Minneapolis, were used for all of the series. The cholesterol in the alpha and beta lipoproteins was measured in several of the series by an adaptation of the total cholesterol method to the fractions separated by paper electrophoresis (23).

In all of the series identity of procedure and analysis was guaranteed by the fact that at least some of the same technical personnel participated in the work in all of the series of studies except those in Malmö and there Dr. Bengt Swahn, who was in charge of the chemical analyses, had just previously worked with the laboratory team in the work in Naples. As a final check on assuring strict comparability of the cholesterol results, a large number of samples were analyzed in duplicate both in Minneapolis and in one of the other field centers—Naples, Cape Town, Bologna. The highly satisfactory agreement in these independent checks in different laboratories has been reported elsewhere (23).

Dietary estimates

The populations considered here were selected for study mainly because their habitual diets cover a wide range of relative fat content with relatively sharp and consistent differences between them as indicated by personal knowledge of the several cultures and by data on food balances of the regions. In most of the studies each man was queried about his personal diet, particularly in regard to the consumption of fats and fatty foods, but these individual data are considered to be adequate only for estimating the relative intakes of fats and to show that the men studied did, in fact, conform to the general dietary pattern of the region.

The values given in Table I, are derived from a variety of sources, as indicated below. Their precision is obviously limited but there is no doubt that, at least, they correctly give the order of fatness of the diets: Minnesota > Malmö > Bologna > Sardinia > Naples > Cape Province Bantu. Further, it is certain that the diet of the Cape Province Europeans is in the same general range of fat proportion as that of the Minnesotans and that the Cape Coloured men eat a diet more like, in regard to fat, that of the men of Sardinia or Bologna.

The Minnesota value in Table I is derived: a) from household surveys in Minneapolis and St. Paul covering 253 families in the winter and 166 families in the spring of 1948, indicating respectively, 41.1 and 43.9 per cent of calories from fats (25) and, b) one-week individual records of total food consumption of 119 middle-aged men, some of whom limited their diet to control weight, in the same cities in 1953 and 1954. The average for this careful survey (7) was 42 per cent of calories from fats but when full allowance was made for fat-trimming at the table and grease adhering to the plates (plate waste) the average was 39.5 per cent fat calories in the food actually eaten. It should be noted that for comparison with the other populations considered, the former figure, *i.e.*, the 42 per cent uncorrected for plate waste, would be more appropriate because allowances for such plate waste are not available for the other populations.

The general order of relationship between the diet of the U. S., Sweden and Italy is indicated in such broad summaries as those of the U. S. Department of Agriculture (26) and the F.A.O. Second World Survey (27) but more useful details are given in the F.A.O. food balance sheets (28) and in the agricultural statistics, supplied by the respective Departments or Ministries of Agriculture, of the countries concerned. These indicate averages for recent years for the U. S., Sweden and Italy, respectively, or 41, 37.5 and 20 per cent of calories from fats.

For Malmö, and the Province of Skåne generally, the true figure may be a trifle higher than the estimated average for all of Sweden. Such a difference is locally thought to exist and a recent survey in Skåne (29) tends to bear this out. Further, this survey shows that the

habitual diets of the different social classes in this part of Sweden are remarkably similar, though, as will be noted later, there may be a tendency for heavy workers to derive a somewhat smaller percentage of their calories from fats than does the rest of the population.

For Bologna, the value listed was obtained by calculation from data provided by Professor Arrigo Poppi and from detailed lower and middle-class dietaries compiled by Dr. Iasonni which agreed, in general, with the local data on food disappearing at the retail level (30). In Italy it is possible to reconstruct a major part of the diet for any town because most foods, including those locally produced by individual farmers, are subject to tax ("imposte di consume") which is enforced at all points of entry to the town. The food "disappearance" data contained in the voluminous statistical reports of the Comunes of Bologna and Naples (30, 31) bear out the difference between the two regions. It is well known in Italy that the diet of Bologna, and of nearby Modena, is much higher in fats than the national Italian average and the region is traditionally known as "Emilia la grasse" (Emilia the land of fats). This is abundantly clear to anyone who alternately lives in Bologna and other Italian cities such as Naples and Cagliari. Besides the much greater use of fat-rich sauces ("Bolognese"), most of the bread is made with about 5 per cent of its dry weight as fat (usually half olive oil and half lard), which is never done in Naples or Sardinia, and the local "pasta" (spaghetti, *etc.*) also differs in that it is often made with eggs and fats. Finally, the food habits recorded by the men actually used as subjects showed that their fat consumption averaged much more than that of the typical Neapolitans studied—more frequent use of butter, fat meat, whole milk cheese, "caffe latte" (hot milk with coffee extract), and more liberal use of olive oil, as well as the habitual use of bread containing fat. Nevertheless, the true average for the subjects studied in Bologna cannot be specified more accurately than being between 27 to 33 per cent of calories from fats.

For Sardinia, many data are summarized by Brotzu (32). The most recent values show a range from 13.6 per cent fat calories for poor laborers to 31.6 for families in reasonably comfortable circumstances in one study and, in another study, averages of 20.9 and 23.9 per cent were obtained for two rural areas in the spring and summer of 1952. These data suggest an average value of around 25 per cent for the urban population and somewhat less for Bacu Abis. In our own surveys on the individuals reported here we concluded that the diets in Cagliari and Bacu Abis, though they resemble the Neapolitan pattern, were richer in fats at the time (Spring, 1955) of our survey. This was indicated, in particular, by the more frequent use of meat, fish and eggs.

For Naples, averages of 18 to 21 per cent fat calories were obtained in two surveys of local mental hospitals (33, 34) by computation from the diets of the Fire Department, and by one-week food consumption records in 1954 of a few of the families of our subject group who were believed to represent the general working class. Further, total food "disappearance" data indicate that Naples corresponds closely to the value of 20 per cent fat calories estimated for all of Italy from food balances. Finally, the results from individual dietary interviews with the Neapolitan subjects conformed to the same pattern repeatedly found for the general population of this area by Professor Gino Bergami—about 70 per cent of calories from cereal products (plain bread and pasta), small portions of meat or fish not oftener than 2 or 3 times a week, fairly liberal amounts of sugar and of fresh cheese made from skimmed milk, fruit and green vegetables several times daily, whole milk only in breakfast coffee, no butter or margarine, much tomato paste, 100 to 200 ml. of olive oil a week, and very small amounts of whole milk cheese ("Parmesan").

The values for Cape Town in Table I are from the household surveys of Batson (35) whose value of 35 per cent fat calories for the local European does not pertain to the upper economic class. It was estimated that the value for the upper economic class of Europeans, who constituted a considerable proportion of the European men studied by us, there, is nearer 40 per cent.

Each subject studied at Cape Town was given a detailed dietary interview by a trained local social worker who was assisted by a dependable Bantu in the interviews with members of that race. This provided reasonably satisfactory figures for the fats and fatty foods but the absolute total calorie consumption from all sources could not be reliably estimated in this way. Accordingly, for some purposes (as in Table V) we believed it more conservative to compare the men in Cape Town in terms of relative fat consumption. The general dietary patterns found in our interviews corresponded well with those recorded by Professor Batson (35) in his studies on sample families in these same populations.

TABLE I

Percentage of total dietary calories supplied by fats in the average diets of the populations studied

Minneapolis, Minnesota	41
Malmö, Sweden	38
Cape Town, Europeans	35–40
Bologna, Italy	29
Cape Town, Coloured	25
Sardinia (Cagliari, Bacu Abis)	22–27
Naples, Italy	20
Cape Town, Bantu	17

Physical activity

For the present purpose the men may be classified according to physical activity into broad groups:

1) *Light work.* This category includes sedentary workers and men whose jobs make very little demand on muscular effort—clerks, shopkeepers, inside watchmen, traders, draftsmen, *etc.* Parenthetically, it was observed that almost no men in any population, in South Africa as elsewhere in our experience, were willing to consider themselves—or to accept the label without much qualification—as being sedentary.

2) *Moderate work.* This category includes men whose jobs infrequently entail physically violent or severe muscular effort but who are fairly active during a good part

TABLE II

Firemen (lines 1, 2, 5), policemen (line 3), firemen and policemen (line 4), studied in 1954 and 1955. Ages 40 to 51 inclusive

Place	N	Mean age	Total cholesterol Mean	S.E.
1. Minneapolis	119	44.2	250.2	±3.8
2. Malmö	38	44.9	237.2	±7.0
3. Bologna	28	45.1	193.8	±7.1
4. Cagliari	29	45.2	193.8	±6.6
5. Naples	76	43.8	169.5	±4.0

of the work day—machine operators, carpenters, painters, light warehouse workmen, janitors, outside watchmen, streetcar servicemen, mechanics, *etc.* Firemen and policemen might be assignable to this "moderate activity" classification but it is useful to consider them separately; they represent a relatively special and homogeneous segment of that class whose activity is light, on the average, but occasionally may be very strenuous. The Minneapolis firemen may be somewhat less physically active than their counterparts in the European cities who tend to have a more regular program of calisthenics and drill. Most of the policemen in these European cities were on patrol duty on foot; only a few of those studied were "desk-bound" or "motorized."

3) *Heavy work.* This category includes men whose jobs regularly involve much heavy lifting and severe muscular effort in steel mills, shipyards, foundries, coal mines, manually handling heavy boxes and barrels in warehouses, loading ship cargoes, road building, quarrymen, *etc.*

RESULTS

Tables II, III, and IV summarize the data for serum total cholesterol in 290 firemen and policemen, 182 men engaged in light work, and 247 men regularly employed in heavy manual labor. For each of the physical activity categories the age of the men is standardized. The data clearly show that the differences between the serum cholesterol averages of the population samples are relatively

TABLE III

Light workers, ages 40 to 59, inclusive

Place	N	Mean age	Total cholesterol Mean	S.E.
Cape Town European	64	48.8	243.8	± 5.9
Malmö	46	49.3	239.4	± 6.2
Bologna	17	47.1	219.9	±15.6
Naples*	22	48.2	164.5	± 7.1
Cape Town Bantu	33	48.0	177.0	± 6.0

* Clerical employees of the city of Naples.

TABLE IV

Heavy manual workers, ages 40–59

Place	N	Mean age	Total cholesterol Mean	S.E.
Malmö	88	49.2	219.1	±3.7
Bacu Abis	59	44.9	181.3	±4.3
Naples	71	49.2	160.2	±3.6
Bantu	29	46.4	147.6	±3.8

independent of the habitual physical activity but, in each activity group, the correspondence of the serum values with the dietary habit is pronounced. For men of the same age and engaged in work of the same general level of physical effort, the group average differences between those on highest fat diets and those on the lowest fat diets are 80.7 mg. per cent for firemen and policemen, 66.7 for light workers and 71.5 for heavy workers and these differences, as well as many other differences within the tables, are highly significant statistically.

The comparisons summarized in Figure 1 are particularly informative because they cover 3 ac-

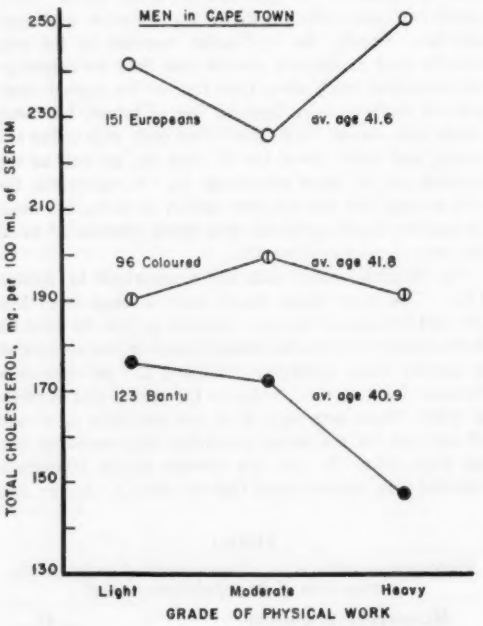

FIG. 1. MEAN VALUES FOR SERUM TOTAL CHOLESTEROL CONCENTRATION, IN MG. PER 100 ML., FOR MEN IN CAPE TOWN, AGED 30 TO 51, CLASSIFIED ACCORDING TO THE PHYSICAL ACTIVITY DEMANDED IN THEIR HABITUAL WORK

tivity levels, graded on the same system, for each of 3 dietary-pattern groups in the same area. It should be observed that the 370 men summarized here are age-matched and were resident in a single urban area with a mild temperate climate where there are no complications of tropical disease or of differential exposure to bitter cold or to extremes of heat. Obviously the differences between the cholesterol values of the Europeans, the Cape Coloured men and the Bantu are not dependent on differences in physical activity and, within the first two of these groups, there is no indication of an effect of the degree of physical activity.

Among the Bantu, however, the men doing heavy manual labor have significantly lower cholesterol values than the other Bantu and the question may be asked as to what this implies. This question becomes more intriguing when it is noted that a similar, though smaller, trend was found in some other populations. For example, heavy manual workers aged 40 to 59 in Malmö averaged 20.1 mg. per cent lower in serum cholesterol concentration than the light workers of the same age in the same place, the difference being statistically significant. On the Island of Sardinia, too, the coal miners of Bacu Abis tended to have lower cholesterol values than their countrymen engaged in moderate or light work in Cagliari.

It is conceivable that this tendency for heavy manual workers to have low cholesterol values is, in fact, related primarily to their high level of physical activity, but a persuasive dietary explanation can also be offered which explains not only the difference between the activity classes of the Bantu but also the lack of differences between the activity classes within the Cape Coloured and the European populations.

Table V summarizes the data on the relative dietary fat consumption for men aged 30 to 59 years in the three populations in Cape Town, classified according to their habitual level of physical work. For each category, Table V gives the mean and its standard error for fat intake expressed as a percentage of the average for the entire series of 468 men. Among the Bantu, the heaviest workers have much lower fat intakes than the other Bantu, but this difference is not evident among the Coloured and European populations.

It should be noted, further, that the data in Table V refer to absolute amounts of fat consumed, with no allowance for the fact that the total calorie consumption is related to the degree of physical activity. But if, as we suspect, the proportion of dietary calories provided by fats is more important in regard to the serum cholesterol level, then it is clear that the inverse relationship between dietary fat and physical activity of the Bantu is even more marked than indicated in Table V. And for the Coloured and European groups it appears that there may be some tendency for the *proportion* of fat in the diet to decrease with increasing physical activity and the corresponding increase in total calories. For example, if we take the reasonable figures of 2,500, 3,000 and 3,500 calories per day to represent the average total energy consumption for calorie balance, respectively, in the light, moderate and heavy work categories, it appears that the proportion of calories from fats is about 20 per cent higher in the diet of the Europeans in light work than in the diet of their fellows in heavy work. But the corresponding figure of the Bantu in light work is more than 100 per cent higher than that for the Bantu in heavy work.

TABLE V

Relative average fat intake, as percentage of the mean intake of all men, in three activity classes of each of three ethnic groups, in Cape Town

Group	Work	No. men	Relative fat intake	
			Mean	S.E.
Bantu	Light	52	75.2	± 6.8
Bantu	Medium	97	74.1	± 5.8
Bantu	Heavy	40	51.2	± 6.8
Coloured	Light	32	104.8	± 4.9
Coloured	Medium	77	115.6	± 4.5
Coloured	Heavy	16	109.3	±10.9
European	Light	78	122.3	± 2.9
European	Medium	57	129.3	± 6.6
European	Heavy	19	143.7	±12.9

Total versus beta lipoprotein cholesterol

In this paper attention is concentrated on the total serum cholesterol. However, it should be noted that the differences between these population groups are equally striking when comparison is made in terms of the cholesterol contained in the beta lipoprotein fraction. In general, the percentage of the cholesterol found in the beta lipoprotein fraction rises with increasing total

cholesterol in these series. For example, the mean percentages of the total cholesterol found in the beta lipoprotein fraction in the serum of men aged 40 to 49, inclusive, in Cape Town are 71.87 per cent for 83 Bantu, 75.45 per cent for 60 Coloured, and 83.28 per cent for 77 European men. In other words, the differences between these ethnic groups are greater in terms of beta lipoprotein cholesterol than in terms of total cholesterol.

Superficially these values may seem to indicate a different relationship than that reported from comparisons of young Nigerian natives with men in the U. S. Mann, Nicol, and Stare (8) concluded that there were large and significant differences in total cholesterol concentrations but that "the serum lipoprotein concentrations of the Nigerians were only slightly lower than those of the North Americans and these differences were of doubtful significance" (*ibid.* p. 1010).

However, it must be observed that in the study by Mann, Nicol, and Stare (8) the beta lipoprotein was estimated by ultracentrifugation and a measurement depending on the refractive index associated with Gofman's fractions "S_f 12–20" and "S_f 20–100." This is not, of course, the same as measuring the beta lipoprotein cholesterol as done here. Further, in their study we note that in regard to the S_f 12–20 fraction, the mean for North Americans was actualy 32 per cent higher than for the Nigerians and that this is significant at the 5 per cent level. It is difficult to establish high statistical significance with relatively small numbers of subjects by the ultracentrifugal method when, as here, the standard deviation, as a percentage of the mean, is as high as ± 50 per cent (N. Americans) or ± 57 per cent (Nigerians).

It may be noted that besides the large differences in the proportion of fat calories in the diets of these populations, the total calorie balance, as shown by relative body weights of the men, varied somewhat, though these differences were relatively small. For example, for ages 30 to 59 years, the mean relative body weights, as percentages of the values for equal age and height given in the standard U. S. Medico-actuarial tables, were 101.5, 96.9, 96.9, and 94.6 per cent, respectively, for men in Cagliari (74 men), Naples heavy workers (106 men), Malmö heavy workers (129 men), and Cape Coloured (124 men).

TABLE VI

*Comparison of serum cholesterol values of men subsisting on different diets but matched for age, relative body weight (on U. S. standards) and physical activity**

Place	Age *years*	Relative *weight*	Cholesterol *(mg. %)* Mean	S.E.
Minneapolis	33.0	103.2	227.7	± 5.9
Naples	33.2	103.8	154.2	± 4.9
Difference	−0.2	−0.6	73.5	± 7.7
Cape Town	47.4	102.1	203.5	± 9.2
Cagliari	47.6	102.8	151.7	± 6.5
Difference	−0.2	−0.7	51.8	±11.3

* Forty-nine Minneapolis firemen are compared, in regard to total cholesterol concentration, with 49 Naples firemen, and 35 moderately active men in Cagliari (Sardinia) are compared, in regard to beta lipoprotein cholesterol, with 35 similarly active white (European) men in Cape Town.

These differences in relative obesity do not account for the serum cholesterol differences observed. Illustrations of the fact that the observed serum cholesterol differences are not dependant on differences in relative obesity or overweight are given in Table VI. Forty-nine Naples firemen were matched in regard both to age and to relative body weight with 49 Minneapolis firemen. The age range was restricted to 29 to 39 years and each Neapolitan fireman was matched within 2 years of age and 3 per cent in relative body weight with a corresponding Minneapolis fireman. It will be observed that these men, all of the same occupation, and averaging within 0.2 year of age and 0.6 per cent of relative body weight, differed by a mean of 73.5 mg. of cholesterol per 100 ml. of serum, the standard error of the difference being only ± 7.73 mg. per cent.

Table VI also shows a comparison of moderately active men in Sardinia with age-activity-obesity mached white men in Cape Town, the beta lipoprotein cholesterol concentration being the variable of focus in this case. Here, too, it is clear that a major difference between the populations in the cholesterol system is not dependent on a difference in relative obesity.

DISCUSSION

The possibility that, in some populations at least, a difference in serum cholesterol concentration between heavy workers as contrasted with sedentary or light workers is related to differences

in the diet, deserves much more investigation. It is surprising to discover that there is extremely little information about voluntary dietary habits in regard to fats of men in different grades of work in the same cultural and socio-economic group. One of the few published sets of data indicated that the percentage of fat calories in the average diet of 10 clerks in Scotland was 35.3 while that for 19 coal miners was 33.5, a slight but significant difference (36).

Dr. M. J. Babcock of the New Jersey Agricultural Experiment Station in New Brunswick, has very kindly supplied us with data obtained in a dietary survey of employees in pharmaceutical factories in his area. Sixty-six men engaged in sedentary and light work were estimated to have an average intake of 3,105 Calories daily, of which 40.39 ± 1.08 per cent were supplied by fats. For 13 men engaged in heavy work in the same factories the average calorie intake estimate was 3,880 Cal. of which 37.04 ± 1.04 came from fats. The difference between the two types of workers was 3.35 ± 1.37, this latter standard error being computed from the pooled variance. This difference is significant at the 2 per cent level of probability ($t = 2.44$; for $P = 0.02$, $t = 2.39$).

After our work in Sweden was finished, a special dietary study was made in Skåne and in a town in Central Sweden by Persson and Nystrand (29) that bears on this point. Samples of three social (and economic) classes were studied. In spite of a high degree of general similarity between the diets of these classes, there was evidence of a consistent trend for the proportion of fat in the diet to rise with rising social class. Class III, in which the heavy manual workers belong, obviously tends to have the lowest proportion of fat calories in the diet. The tendency is for the heavier workers to get the extra calories used in their work by adding carbohydrate (potatoes, sugar, bread) to their diet. For example, there were no or only trivial differences between the averages for the 3 social classes in regard to the amounts of butter, margarine, milk, cream, cheese and eggs consumed per standard family, or in the frequency of eating meat and fish. But social group III ate much more bread, potatoes and sugar than Class II (which includes office workers and persons in light activity in factories).

Another item of possible significance is the consistent difference reported from many parts of the world between urban and rural people. The latter, of course, may generally be assumed to be considerably more active, physically, than the former. In Japan, as an example of a country where the diet is generally very low in fats, the estimate for 1950 is that the urban population gets only 9.7 per cent of its calories from fats while the rural population gets only 6.4 per cent of the calories in this form, *i.e.*, only two-thirds as much in proportion. And in the United States, an example of a high-fat diet country, the estimates (1942) for urban families were 42 per cent of calories from fats and 37 per cent for rural diets (25). The same trend is seen in the surveys on the Island of Sardinia (32).

These data show we believe, that the large differences in serum cholesterol concentrations observed when populations of different dietary habits are compared, are not the result of differences in physical activity. This is not meant to imply, however, that physical activity has no effect on cholesterol metabolism. This question needs, and deserves, special study.

SUMMARY

1. Data on total serum cholesterol are presented for samples of men, classified by age and physical activity, in Minnesota, Malmö, Sweden, Bologna, Naples, the Island of Sardinia and three ethnic groups in Cape Province, South Africa. Data on the cholesterol in the beta lipoprotein fraction in the serum are presented for some of these groups.

2. Differences in physical activity do not explain the large differences in serum cholesterol found when groups with different dietary habits are compared.

3. Within some populations there is a tendency for men in heavy manual labor to have somewhat lower serum cholesterol values than the other men in the population. Among the Bantu, who show this tendency most prominently, it was found that the men in heavy work consume diets lower in fats than the other Bantu and there is other evidence pointing to a general tendency for the composition of the diet to be related similarly to the habitual level of physical activity.

4. It is concluded that the habitual diet, and especially its fat content, has much more influence

than the physical activity, *per se*, on the concentration of total cholesterol and beta lipoprotein cholesterol in the blood serum.

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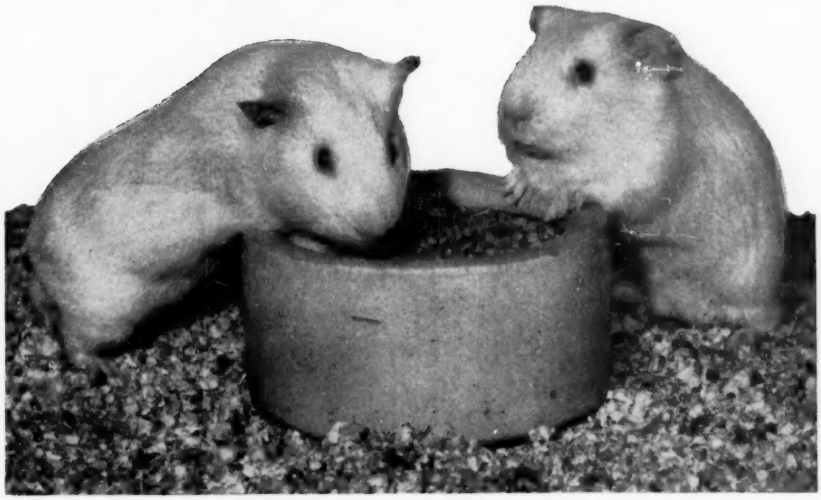

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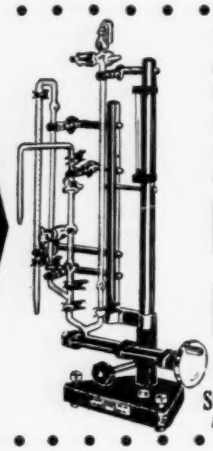

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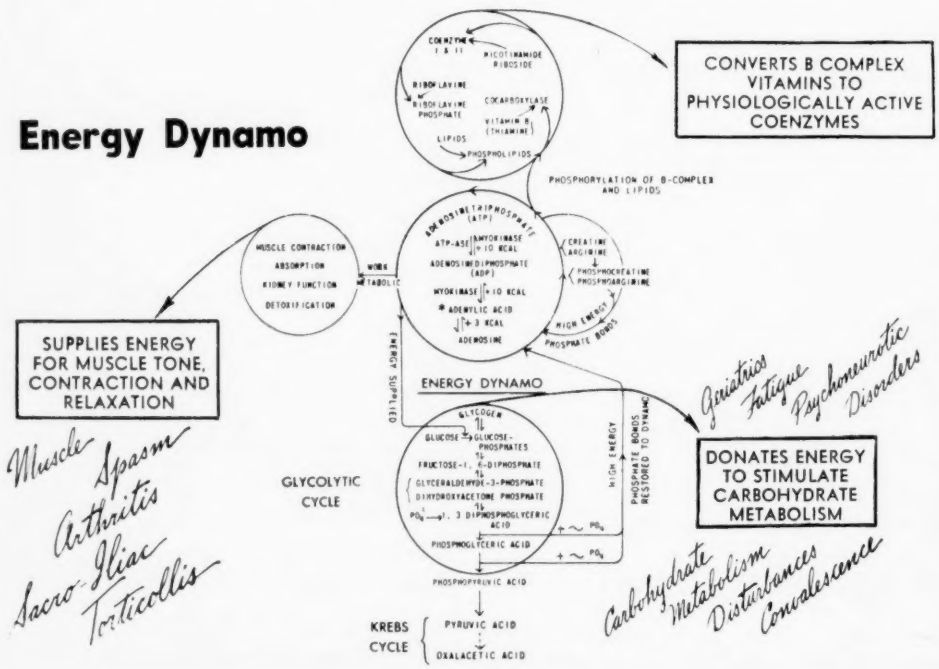

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